The roles of F-actin and integrin-like proteins in hyphal tip growth

A thesis

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Abstract

This thesis presents an investigation into the role of F-actin and integrin-like proteins in tip growth in the oomycete *Achlya bisexualis*. Tip growth is a complex process that involves localised extension at the tips of hyphae. It has been suggested that F-actin plays a number of roles in the process; these include the provision of both resistive and protrusive forces and the delivery of vesicles to the sites of growth. Any role that includes the provision of force requires the attachment of F-actin to a structure such as the cell wall.

To understand fully the role of F-actin in tip growth requires knowledge of its location within hyphae. Using a combination fixative of methylglyoxal and formaldehyde it was found that two distinct distributions were present. In around one half of hyphae examined a new structural feature, the F-actin depleted zone was observed. The remaining hyphae had complete F-actin caps. It is suggested that this depleted zone represents an area where localised tip yielding occurs due to a decrease in the resistive force provided by F-actin. It may also represent an area where exocytosis of wall vesicles occurs.

The attachment of F-actin to the cell wall may occur via proteins that are similar to the integrins of animal cells. These proteins are sensitive to peptides that contain the sequence Arg-Gly-Asp (RGD) and thus such peptides have often been used to indicate the presence of integrins or integrin-like proteins. The peptide Arg-Gly-Asp-Ser (RGDS) was found to reversibly affect the shape of growing hyphal tip, slow the growth rate and affect the organisation of F-actin in a dose dependent manner. Furthermore, it affected the patterns of protoplasmic retraction that were observed as hyphae were plasmolysed. This latter observation suggests that integrin-like proteins may be responsible for wall membrane attachment. These attachments are sites where F-actin is present and thus the integrin-like proteins could provide an attachment site for F-actin that allows roles in tip

growth that include the provision of, or resistance to, force. Furthermore, using the technique of fractal geometry, RGDS, despite slowing growth, was found to have no effect on the morphology at the edges of mycelia. Agents that slow growth typically increase branching and thus affect such morphologies. This suggests that integrin-like proteins may play a role in coupling the processes of tip growth and branching or they may inhibit branching and thus negate the normally increased rate that is observed with the slowing of growth.

Acknowledgements

First of all I would like to thank my supervisors, Dr. Ashley Garrill and Dr. Sandra Jackson (Department of Biological Sciences, University of Canterbury) for their constructive guidance, advice through the years and especially helping to correct my thesis to an academic and readable shape.

I would also like to thank the University of Canterbury for a doctoral scholarship (University of Canterbury Doctoral Scholarships), which financially supported me while pursuing my degree.

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Finally, a special thanks to Dr. Mung-Shiung Shieh, the president of Shih Chien University, for giving me an opportunity for study leave. And also thanks to my mother for her encouragement and understanding.

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Abbreviations

ANOVA	Analysis of Variance
AP	Alexa 488 phalloidin
CaM	Calmodulin
dd H_2O	Double distilled water
DIC	Differential interference contrast
ECM	Extracellular Matrix
FA	Formaldehyde
F-actin	Filamentous actin
FAK	Focal adhesion kinase
GGR	Glycine-glycine-arginine
HEPES	N-2-hydroxyethyl piperazine N'-2-ethane sulphonic acid
MG	Methylglyoxal
PIPES	Piperazine-N N'-bis (2-ethane sulphonic acid)
PYG	Peptone-yeast extract-glucose medium
RGD	Arginine-Glycine-Aspartic acid
RGDS	Arginine-Glycine-Aspartic acid-Serine
RP	Rhodamine-conjugated phalloidin
SES	Standard extracellular solution
TEM	Transmission electron microscopy
TPA	Tetrapentylammonium chloride

Chapter 1
Introduction

1.1 Tip growth and morphogenesis

Tip growth, apically localized cellular expansion, is the hallmark of hyphal organisms such as oomycetes and the fungi and involves many cytoplasmic activities that include cell wall synthesis, cytoplasmic migration and organelle movement. It has been observed in a variety of additional cell types such as pollen tubes, root hairs, and algal rhizoids (Heath, 1995). Morphogenesis of hyphae is thought to result from a balance between regulated extension of the tip and a protrusive force that enables this extension. This force is often assumed to be turgor pressure, however, the relationship between turgor pressure and growth rate is complex which suggests that additional mechanisms of force generation may be present (Kaminskyj *et al.*, 1992a). Indeed, two species of water molds are able to grow even under in the absence of detectable turgor pressure (Money & Harold, 1993) and fungal hyphae that have no cell wall and hence that are unable to generate turgor pressure can grow with a normal shape (Katz & Rosenberger, 1970). These observations indicate that there is no simple relationship between turgor, cell wall synthesis and growth.

1.2 Cell wall synthesis and tip yielding

Synthesis of the apical hyphal wall involves characteristic 'wall vesicles' (Heath, 1994) which occur most abundantly in the apex of a growing hypha and also in localised sub-apical regions, which are presumably sites of branch initiation. These vesicles are thought to contain enzymes and precursors for the biosynthesis of new plasma membrane and cell wall. They are manufactured in Golgi bodies (or

dictyosome) that are scattered throughout the cytoplasm, from which vesicles are transported vectorially to the extending tip where they accumulate before they are exocytosed (Gooday, 1983; Wessels, 1986; McKerracher & Heath, 1987; Wessels, 1990; Heath, 1990; Harold, 1994). Autoradiographic studies have confirmed that a fungal hypha grows by localized apical wall formation with a sharply descending gradient of wall synthesis radiating from the apex (Bartnicki-Garcia & Lippman, 1969; Gooday, 1971). Electron microscopy studies have shown an accumulation of cytoplasmic vesicles, which have been implicated in such wall formation at the extreme hyphal tips of fungi (Grove & Bracker, 1970; Heath *et al.*, 1971; Heath *et al.*, 1985). Furthermore the hypothesised existence of a wall-vesicle-generating apparatus or vesicle supply centre has been shown in computer simulations to be capable of generating hyphal shapes (Bartnicki-Garcia *et al.*, 1989). However, such computer models are controversial and may not account for growth in the oomycetes where dense accumulations of vesicles are not present.

Any consideration of extension at the tip also needs to take into account gradients of wall properties in the hyphal apex that are pertinent to wall yielding, e.g. elasticity/rigidity, plasticity, or polymer cross-linking. These have been cited by some as essential to morphogenesis (Bartnicki-Garcia *et al.*, 1989; Wessels, 1990) and a means of controlling rates of growth but other workers have criticised these ideas as they place control external to the cytoplasm (Heath, 2001). In the latter critique, emphasis is now placed on the role of the actin cytoskeleton, as well as the cell wall, in regulating tip yielding.

1.3 Turgor pressure

As detailed in Section 1.1, turgor pressure is still widely regarded as fundamental to tip growth. It is thought that pressure on the cell wall is needed to force the expansion of the hyphal tip as the pressure forces wall polymers apart. Such a mechanism may not be universal, however as hyphae of the oomycetes *Saprolegnia ferax* and *Achlya bisexualis*, when grown on media supplemented with 250mM sucrose experienced a reduction of turgor pressure (<0.02 MPa), yet increased the rate of extension (indeed to rates that were faster than normal rates of extension) (Money & Harold, 1993). It

was found that as the osmotic pressure in the medium increased, the decline in turgor was paralleled by a reduction in cell wall strength. The hyphae may have adapted to turgor loss by reducing the strength of their cell walls, and thereby enabled the wall to yield to the reduced pressure (Money & Harold, 1993). In *Saprolegnia ferax* a correlation between the activity of secreted endoglucanases (1,4-β-D-glucanhydrolase) and tensile strength of the walls has been reported (Money & Hill, 1997). This is consistent with the idea that part of the wall-loosening mechanism in these organisms involved the action of these enzymes (Money & Hill, 1997). Furthermore *A. bisexualis* has recently been shown to be incapable of turgor regulation (Lew *et al.*, 2004). For these reasons, in certain organisms turgor can no longer be regarded as the sole determinant of cell expansion, with an implicated additional role for enzyme activity.

In addition to the cell wall, as described above, it has been suggested that the actin cytoskeleton may also play a role in supporting the expanding plastic apex. Thus control of tip yielding may also involve dynamic rearrangement of the actin cytoskeleton. Actin may be multifunctional however as it is also thought to play a role in several other aspects of apical extension such as the process of cell wall synthesis, cytoplasmic migration, and the movement and positioning of organelles (Bachewich & Heath, 1998; Heath, 1990; Jackson & Heath, 1990a; Jackson & Heath, 1993).

Extensive arrays of actin filaments have been seen in hyphal tips and other wall-growing regions of fungal cells (Adams & Pringle, 1984; Hoch & Staples, 1985) (Heath, 1987) and it has been proposed that they pull the cytoplasm in tipward direction (McKerracher & Heath, 1987). Peripheral F-actin network may also influence the tip-high gradient of stretch activated channels in the growing tip of *S. ferax* hyphae (Levina *et al.*, 1994).

As oomycetes have been shown to grow in the absence of measurable turgor there have also been arguments that actin may provide a protrusive force at the tips of hyphae in a manner similar to protrusion of pseudopodia in animal cells. Such a mechanism would necessitate the existence of cytoskeletal cell wall linkages similar

to those of animal cells (Heath, 1994). The components of these linkages are considered further below.

1.4 Organization of actin

1.4.1 Actin distribution in hyphae and its role

Filamentous or F-actin is concentrated in the growing tips of hyphae and buds of yeast, such as *Uromyces phaseoli* (Hoch & Staples, 1983), *Paxillus* sp., *and Suillus* sp. (Salo *et al.*, 1989), *Saccharomyces cerevisiae* (Adams & Pringle, 1984; (Kilmartin & Adams, 1984), *Saprolegnia ferax* (Heath, 1987), *Sclerotium rolfsii* (Roberson, 1992), *Allomyces macrogynus* (Srinivasan *et al.*, 1996). Similar localisation has also been reported in other tip-growing organisms e.g., pollen tubes of the *Gymnosperm picea abies* (Norway Spruce) (Lazzaro, 1996). In hyphal organisms, there are typically two patterns of F-actin observed: plaques and fibrils that sometimes associate to form cables. Usually the distribution of F-actin is peripheral. The actual distribution of plaques and cables of F-actin may differ among species, but as actin filaments are very labile, these variations in actin distribution may be due to different fixation methods used in the various studies (Heath, 1987). Heath (1987) described the actin distribution in chemically fixed hyphae of *Saprolegnia ferax*; at the tip there was a characteristic fibrillar cap with plaques and cables in the more subapical regions. This contrasts however with other tip growing cells such as pollen tubes and root hairs in which an actin clear zone is present at the extreme tips of cells. This suggests that we either may not know the true distribution of actin in hyphae or that no one model can explain tip growth in the various cell types that grow by this means.

Studies of actin distribution have used several different methods to fix and stain the hyphae. In any such study, it is important to preserve the structural organization of the F-actin as close as possible to that of the living cells. Possibly the best way is to introduce a label into living cells, which upon subsequent observation should reveal the dynamic nature of the F-actin. The only report of imaging of living oomycete hyphae used the technique of electroporation to introduce fluorescently labeled rhodamine Phalloidin into growing hyphae. These hyphae showed an actin depleted zone at the tip but poor staining in subapical regions (Jackson & Heath, 1990b) led to

the suggestion that not all populations of actin can be detected in this way. Because of this problem, hyphae have tended to be fixed by using either chemical or rapid freeze fixation rather than using electroporation. In these studies, observations have to be made with the assumption that the structural organization of actin is faithfully preserved by those fixations.

Aldehydes have been widely used as fixatives due to their ability to cross-link proteins (Glauert & Lewis, 1998). Glutaraldehyde is typically regarded as the best cross linker as with two functional aldehyde groups, it can rapidly and irreversibly react with a number of amino acids. Its use is problematic however as it is itself fluorescent, thus causing higher background, and poor contrast. Formaldehyde is another possibility, it has little auto-fluorescence and penetrates tissue more rapidly than glutaraldehyde, but it forms fewer and less-stable cross-links. In choosing the chemical fixatives, a balance of improved contrast and preservation of structural information, is an important consideration. Both of these are important if the true nature of the actin cytoskeleton in hyphal organisms is to be deciphered.

The multi-functionality of F-actin, as described above, is thought to arise from its interactions with a large array of actin binding proteins. If it is to function in regulation of tip extensibility and/or the provision of protrusive forces, it is likely to interact not just with actin binding proteins but also with molecules that link the cytoskeleton with the cell wall. Such linkages would be necessary to enable the provision of a resistive or protrusive force to be consistent with Newton's third law of motion (For every action there is an equal and opposite reaction). Using the framework of animal extracellular matrix (ECM)-cytoskeletal linkages there have been suggestions that molecules similar to the integrins may be involved (Heath, 2001).

1.5 Integrins

1.5.1 Structure

Integrins, dimerized single-transmembrane proteins, are composed of non-covalently linked α and β polypeptide chains (Figure 1.1). There are over 20 different integrin-

isoforms that heterodimerize from among 16 alpha and 8 beta subunits (Horwitz, 1997; Hynes, 1992). Each integrin has its own binding specificity and signalling properties. The subunits contain extracellular, transmembrane, and intracellular segments. The large extracellular part of both α and β subunits bind a variety of ligands such as specific proteins of ECM and of the surfaces of other cells. These ligands often contain Arg-Gly-Asp (RGD) sequences (Ruoslahti & Pierschbacher, 1987). The short cytoplasmic domain of β integrin subunit anchors the cytoskeleton to the plasma membrane via intermediary adaptor proteins.

1.5.2 The importance of integrin

As detailed above, tip growing cells need to regulate tip extensibility and possibly to exert a protrusive force by some means other than turgor. To be consistent with Newton's third law of motion then if actin is to facilitate such a role it must have sites of attachment to the plasma membrane or cell wall. Proteins that are similar to the integrins have the ability to carry out such a role but in addition they may also enable bidirectional signalling or cross-talk between the cell wall and cytoplasm.

In metazoan cells integrins are thought to mediate the interaction of cells with the surrounding ECM, cell adhesion, a process that is essential for anchorage, and act as cues for cell migration and signals for growth and differentiation (Etzioni, 1999; Hynes, 1992). Integrins are components of both "outside-in" and "inside-out" signalling systems. Through the outside-in signalling pathway, binding of extracellular-matrix proteins to integrins alters gene expression and affects cellular proliferation and differentation. The inside-out signal transduction system induces conformational changes in the integrin heterodimer and substantially increases adhesiveness to the ECM (Etzioni, 1999). Integrin signalling is very complex, and is of extreme importance. A brief summary of some of the known signalling cascades generated from integrin activation is showed in Figure 1.2.

1.5.3 Focal adhesion

When integrins bind to the components of the ECM such as the matrix proteoglucan, fibronectin, and collagen this leads to clustering of integrin receptors and the formation of focal adhesions. Integrins also bind to soluble ligands such as

fibrinogen, and receptors on adjacent cells, leading to cell aggregation. Focal adhesion plaques contain intracellular cytoskeletal components, such as talin, paxillin, and tensin, which tether them to actin bundles (Figure 1.3). Focal adhesions (Alberts *et al.*, 2002) play a key role in animal cell motility (Figure 1.4). Cell-movement occurs by the formation and breaking of integrin-mediated attachments to a matrix (Figure 1.5).

In walled cells, similar adhesions between the plasma membrane and cell wall, which may be thought of as analogous to the ECM, have been reported. When such cells are plasmolysed, some cytoplasm and plasma membrane may remain attached to the cell wall (Attree & Sheffield, 1985; Henry *et al.*, 1996; Kagawa *et al.*, 1992; Kaminskyj & Heath, 1995; Oparka, 1994). This has led to the suggestion that these sites represent areas where integrins or integrin-like molecules are linking the cytoskeleton to the cell wall. Their possible involvement in adhesion is further supported in some studies by the effects of the addition of RGD-peptides, which are known to disrupt focal adhesions in animal cells (Henry *et al.*, 1996; Schindler *et al.*, 1989). These are discussed at greater length later in the thesis.

Integrins or some analogous molecules have been reported in hyphal organisms although as detailed later this is still an area of some controversy (Correa *et al.*, 1996; Gale *et al.*, 1996; Kaminskyj & Heath, 1995; Marcantonio & Hynes, 1988). Where they have been reported they are located at the plasma membrane and they show a tip-high concentration gradient that is consistent with a role in growth (Kaminskyj & Heath, 1995). Furthermore the abundance of integrin-containing adhesions and F-actin varies with the growth of hyphae and other life cycle stages (Bachewich & Heath, 1999; Bachewich & Heath, 1997a; Kaminskyj & Heath, 1995) and peptide inhibitors selective for integrin-mediated adhesions influence the behaviour of, at least, some hyphae, although apparently not tip growth (Correa *et al.*, 1996). Further evidence for plasma membrane-cell wall adhesions has been derived from the observation that the incipient plasmolysis method of turgor pressure measurement typically gives higher values than other methods (Harold *et al.*, 1996; Kaminskyj *et al.*, 1992b), but as detailed in chapter 4 this again is still an area of some controversy

and much more research is required to address the presence of integrins in hyphae and a role for these molecules in tip growth.

1.6 Model systems

The oomycetes have been used as an experimental system to study tip growth because they are large, fast growing and easily maintained. As organisms that are evolutionarily distant from the other group of hyphal organisms, the fungi, they also provide a means for studies of convergent evolution of the hyphal growth form (Alexopoulos *et al.*, 1996).

1.6.1 Achlya bisexualis

Achlya bisexualis is an oomycete and a member of the Saprolegniaceae (which are commonly known as the watermolds). *Achlya* is aquatic and saprobic, and can be isolated from freshwater and soil. They have a definite cell wall, but do not have chlorophyll, and are usually non-motile, although they may have motile reproductive cells, and they reproduce by means of spores (Alexopoulos *et al.*, 1996). The oomycetes are generally characterized by having zoospores with two flagella approximately equal in length but different in morphology; one is the whiplash type and the other the tinsel type (Alexopoulos *et al.*, 1996).

The thallus is well developed, eucarpic, and composed of coenocytic hyphae containing numerous nuclei. Generally, they are aseptate, unless septa are formed in the mycelium just below the reproductive organisms and locate between the somatic hyphae and the reproductive organisms.

Glucose is the source of carbon. *Achlya* is unable to utilize nitrites. Organic N is supplied in the form of peptone or any one of the amino acids in the medium (Alexopoulos *et al.*, 1996). They are able to synthesize all the vitamins that are required for growth. Inorganic growth requirements include Mg, Ca, Zn, Mn, Fe, and S. Sulphates apparently cannot be utilized, but S may be conveniently supplied in organic forms as cysteine, cystine, glutathione, or methionine. A pH range from 4.0-6.0 has been found to be optimal for the growth (Alexopoulos *et al.*, 1996).

1.6.2 Life history

According to whether hyphae penetrate into their substratum or not, there are two types of hyphae in mycelia of *Achlya*. One is the rhizoidal hypha, which enter the substratum, and serve to anchor them to substratum and to absorb nutrients. The other is the somatic hypha, which comprises the mass of profusely branched hyphae on the outside of the substratum, and forms a visible colony of the organism and can project above the surface of the medium and thereby constitute the aerial mycelium. Under favour conditions the hyphae put forth branches, whose continued proliferation gives rise to a radial colony (on solid medium) or to felted mycelium (in liquid) (Harold, 1994). Under certain circumstances, such as low nutrient medium; low salt medium, the apices of somatic hyphae develop into sporangia that eventually produce asexual zoospores, which constitutes the organisms asexual life cycle (Figure 1.6). There is also a sexual life cycle that is coordinated by steroid hormones (Alexopoulos *et al.*, 1996).

1.6.3 Hyphal Structure

A key attribute of tip growing cells such as hyphae is morphological polarity. Thus hyphae are polar cells and constitute a useful model system for studies of the development of and the maintenance of polarity. There are typically no large organelles found 2μm from the tips of hyphae. Mitochondria become abundant immediately below this region. Nuclei occur below the main mitochondrial zone. Most of the endoplasmic reticulum has ribosomes attached to the cytoplasmic side of the membranes and thus may be termed rough endoplasmic reticulum. Golgi bodies or dictyosomes are typically composed of 4 or 5 flattened cisternae with their forming faces adjacent to either the nuclear envelope or a cisternum of the endoplasmic reticulum. Microtubules permeate hyphal cytoplasm, running in relatively straight lines for many micrometers parallel to the long axis of the hypha (Heath & Kaminskyj, 1989).

1.7 Summary

The process of hyphal tip growth is likely to arise due to a balance between regulated tip yielding and the provision of a protrusive force. In the oomycetes, the F-actin cytoskeleton has been implicated in playing a role in both of these processes. F-actin typically forms a dense actin cap in the apices of oomycete hyphae although this differs from other tip growing cells such as pollen tubes and root hairs where there is an actin clear zone at the apex. This raises the question of how well the actin cytoskeleton has been preserved in previous studies. Knowledge of the F-actin distribution in hyphae is crucial to any study of its role in the tip growth process.

If F-actin plays any role in force resistance or provision then to be consistent with law's of motion the cytoskeleton should be linked in some way to the cell wall. Such linkage may occur by proteins similar to the integrins of the metazoa. These are transmembrane proteins that bind adhesive proteins of the extracellular matrix and various intracellular cytoskeletal complexes to form focal adhesion. These protein assemblies play important roles in modulating cell adhesion and inducing cell shape changes that are involved in cell spreading and locomotion and help to integrate responses of cells (e.g., movement, growth, differentiation) to changes in the environment. The presence of integrins in oomycetes (and indeed in all non-metazoan cells) is a matter of controversy and so is an area in which further research is needed. Such work will lead to a clearer understanding of the role of F-actin and associated molecules in tip growth.

1.7.1 The objectives of this study

In order to understand better the role of F-actin and associated molecules in tip growth the following objectives were set which form the basis of this thesis:

1. To investigate the effect of various chemical fixatives on the actin distribution in hyphae
2. To investigate the effect of RGD containing peptides, that disrupt integrin-ECM interactions in animals, on hyphal tip growth, hyphal morphology and actin distribution

3. To investigate the effect of RGD containing peptides on wall membrane attachments

4. To investigate the effect of RGD containing peptides on the morphology of mycelia as determined using fractal analysis.

These studies were carried out using the oomycete *Achlya bisexualis*.

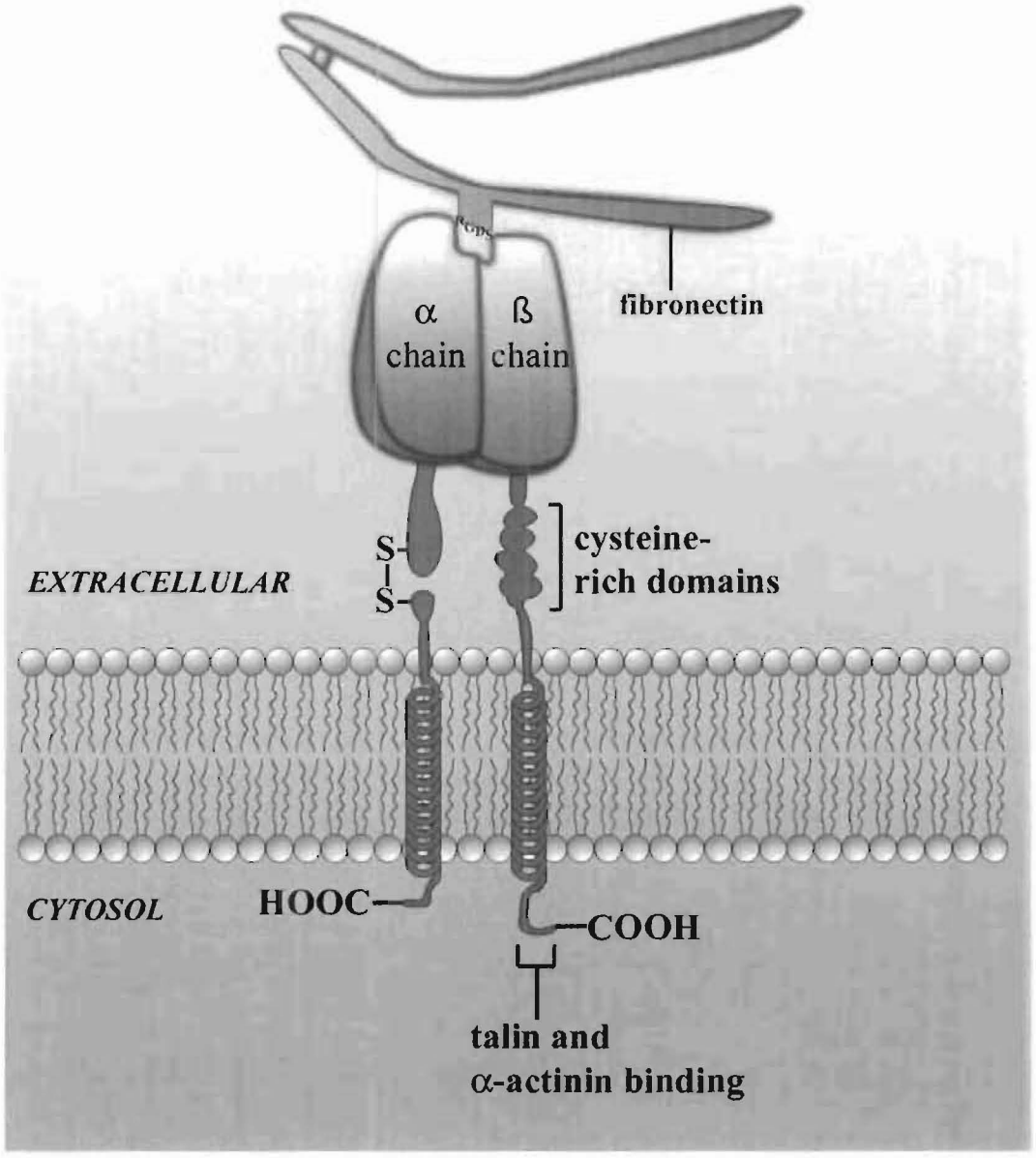

Figure 1.1 The structure of integrins

Integrins are composed of alpha and beta polypeptide chains that link noncovalently. The subunits contain extracellular, transmembrane, and intracellular segments. The extracellular segments bind a variety of ligands such as specific proteins of the ECM, fibronectin and of the surfaces of other cells. These ligands often contain Arg-Gly-Asp (RGD) sequences. The intracellular segments link to actin binding proteins such as talin and α-actinin. Redrawn from Lodish *et al.* (1995)

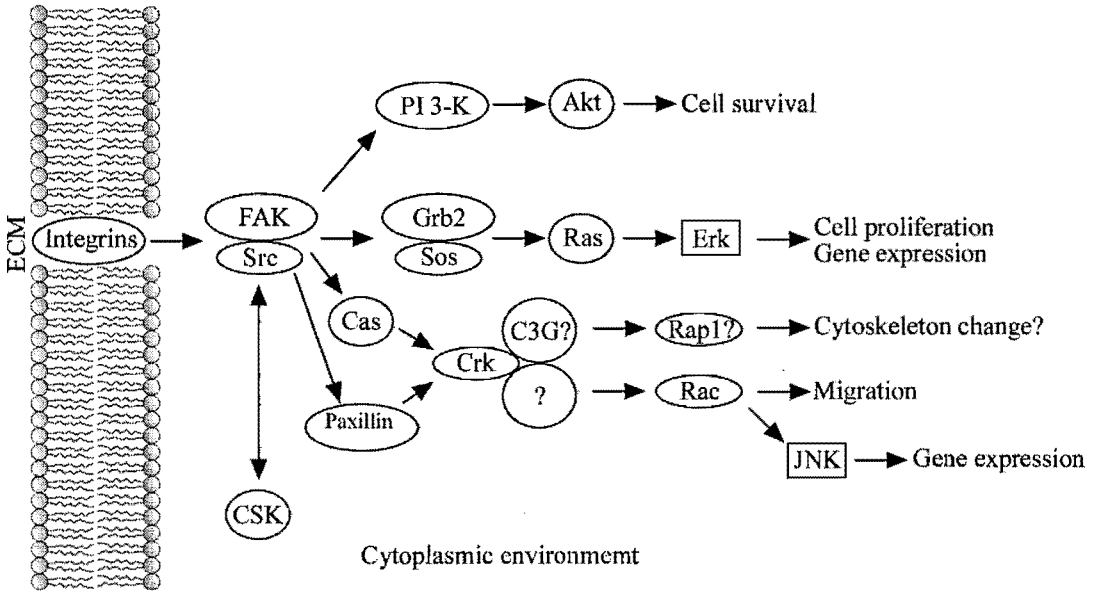

Figure 1.2 Integrin signal pathways through the FAK-Src complex

A putative model for how activation of downstream signalling pathways leads to change in cellular function. As a result of the tyrosine phosphorylation, Focal adhesion kinase (FAK) and Src kinase and the docking proteins residing in focal adhesions form a network of protein-protein interactions that connect a multiple downstream biochemical-signalling pathways. Redrawn from Vuori (1998).

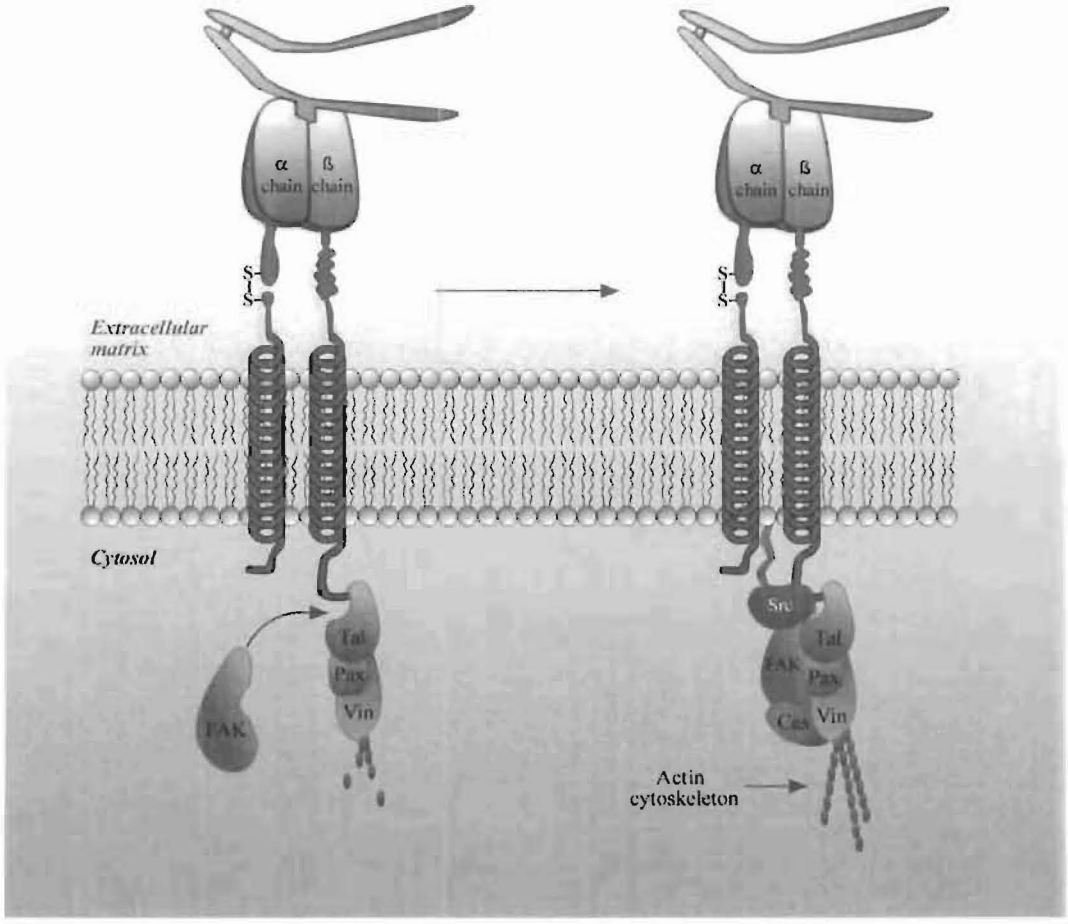

Figure 1.3 Matrix binding promotes integrin clustering and association that in turn promotes further integrin clustering and matrix organization

RGD, Arg-Gly-Asp integrin-binding motif; Tal, talin; Pax, Paxillin; Vin, vinculin; FAK, focal adhesion kinase; Src, cytoplasmic tyrosine kinases. Redrawn from Giancotti (1999).

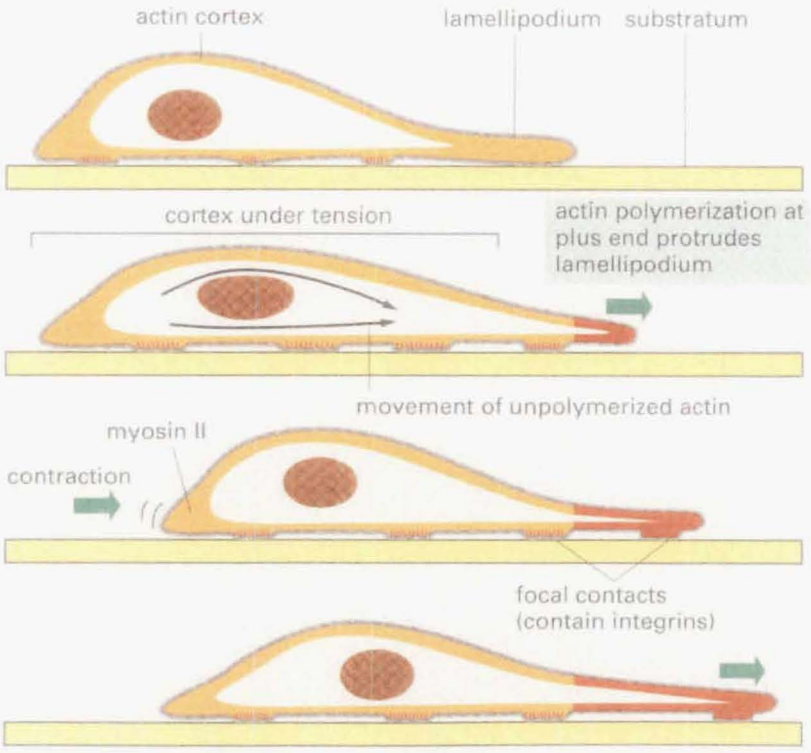

Figure 1.4 A mode of how forces generated in the actin-rich cortex move a cell forward

The actin-polymerization-dependent protusion and firm attachment of a lamellipodium at the leading edge of the cell moves the edge forward (green arrows at the front) and stretches the actin cortex. Contraction at the rear of the cell propels the body of the cell forward (green arrows at the back) to relax some of the tension (traction). New focal contracts are made at the front, and old ones are disassembled at the back as the cell crawls forward. The same cycle can be repeated, moving the cell forward in a stepwise fashion. Alternately, all steps can be tightly coordinated, moving the cell forward smoothly. The new polymerized cortical actin is showed in red. Reproduced from Alberts *et al.* (2002).

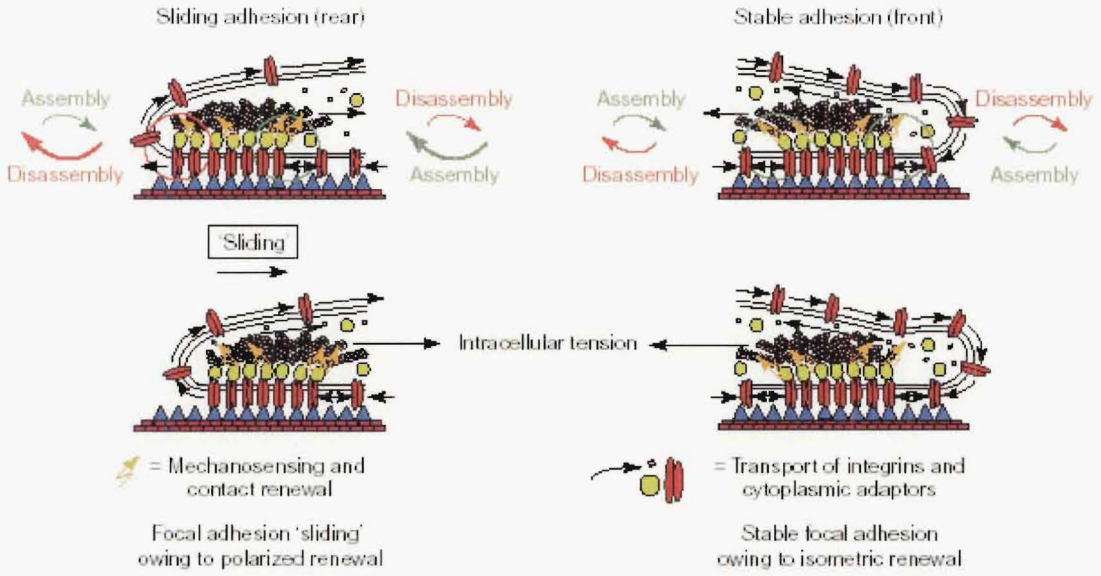

Figure 1.5 A schematic view of the proposed mechanism of sliding caused by a polarized renewal of high-density focal adhesion

The focal adhesion is linked to the extracellular substrate (blue) via integrin heterodimers (red) embedded in the plasma membrane (thin lines) and to the actin cytoskeleton (purple) via various adaptor proteins (green).

High-density focal contacts demonstrate continuous dispersal of old (Disassembly, red) and polymerization and aggregation of new (Assembly, green) focal adhesion components. Local differences in the cellular traffic of these building blocks along the polarized microtubule network creates an imbalance between disassembly and assembly in focal adhesions localized to the retracting rear of migrating cells. This imbalance results in a polarized renewal of focal adhesions, giving the impression of 'sliding' (left-hand side). At the cell front, however, defined by the presence of an advancing lamellipodium, integrins and adaptor proteins ready to become incorporated into the renewing focal adhesion have similiar access to all sides to induce a nonpolarized renewal that does not result in sliding (right-hand side). Copied from Wehrle-Haller & Imhof (2002).

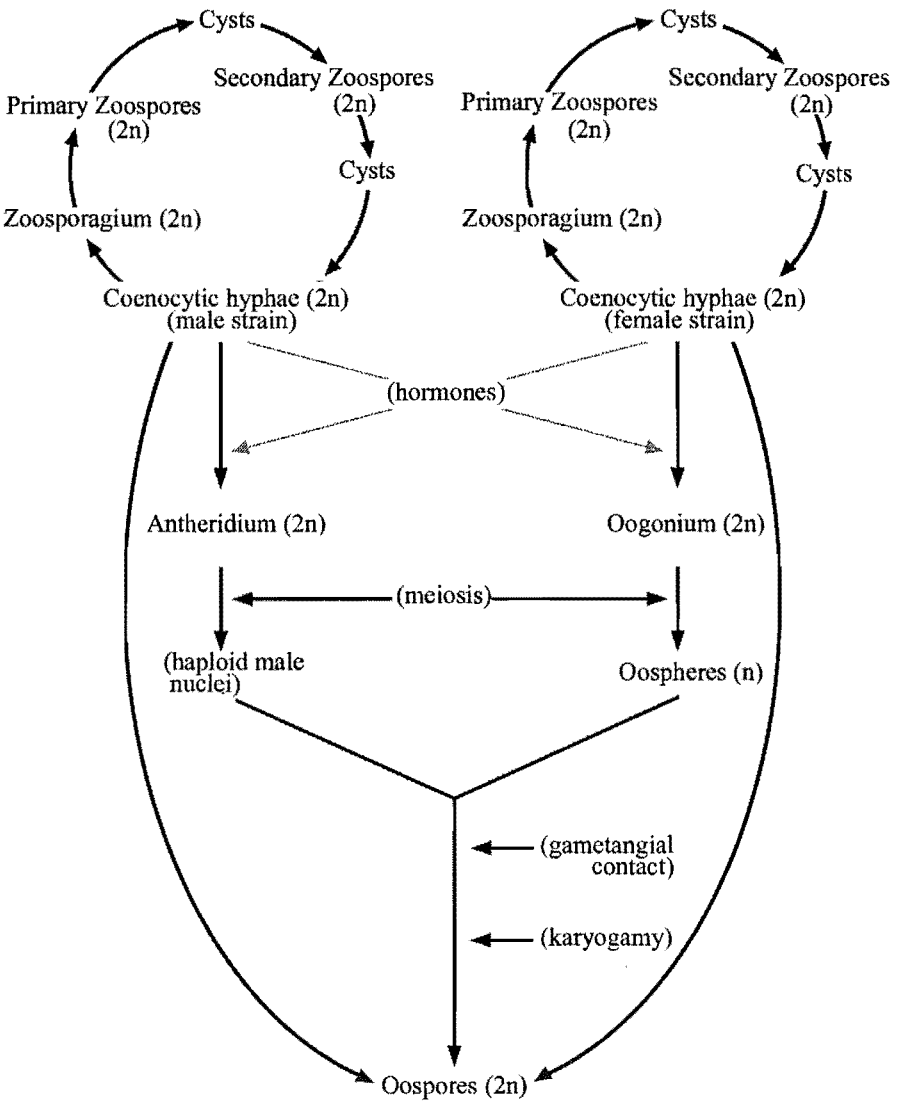

Figure 1.6 Life cycle of *Achlya bisexualis*

Chapter 2

Two distinct distributions of F-actin are present in the hyphal apex of the oomycete *Achlya bisexualis*

2.1 Permission Letter (p19)

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Two Distinct Distributions of F-actin are Present in the Hyphal Apex of the Oomycete *Achlya bisexualis*

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We show that two distinct distributions of F-actin are present in the hyphal apex of the oomycete *Achlya bisexualis*, that have been chemically fixed with a combination of methylglyoxal and formaldehyde and stained with Alexa phalloidin. In approximately one half of the hyphae examined, an F-actin depleted zone within the apical F-actin cap was observed. The remaining hyphae had a continuous apical cap. In live, growing hyphae two types of cytoplasmic organization were observed at the tips, one in which a clear zone was present which may correlate with the F-actin depleted zone, and one where no such clear zone existed which may represent the continuous cap. We suggest that the F-actin depleted zone may be a structural component of the actin network in a subpopulation of oomycete hyphae and may be comparable to similar F-actin depleted zones at the apices of other tip growing cells such as pollen tubes and root hairs. This observation has implications with regard to models of hyphal extension. Hyphae fixed with formaldehyde alone showed continuous apical F-actin caps. Our ability to resolve the F-actin depleted zone likely reflects the cross-linking capabilities of methylglyoxal. The methylglyoxal–formaldehyde combination fixative gave more stained hyphae, brighter staining and more complete staining of F-actin compared to formaldehyde alone.

Keywords: *Achlya bisexualis* — Actin — F-actin depleted zone — Oomycete —Tip growth.

Introduction

Tip growth is a form of cell extension characteristic of a number of plant and fungal cells that include oomycete and fungal hyphae, pollen tubes, algal rhizoids and root hairs. It is a complex process in which growth is restricted to the apex or tip of the cell, this localization giving rise to tubular cells. Models of tip growth typically espouse the importance of the actin cytoskeleton, which is thought to play a number of roles, including acting as a morphogenic factor (Picton and Steer 1982), acting as a determinant of polarity (Vidali and Hepler 2001), acting as a provider of structural rigidity (Jackson and Heath 1990) and enabling the delivery, filtering and retention of exocytotic vesicles to the growing tip (Miller et al. 1999, Geitmann and Emons 2000).

Investigations of the actin cytoskeleton and its role in tip growth are reliant upon our ability to faithfully observe the structural organization of actin microfilaments. In oomycetes these appear to form an apical cap with a subapical arrangement of fibrils and plaques (Heath 1987). One particular facet that is still poorly understood is a reported F-actin depleted zone that appears to be present in the apex of a number of tip growing cells (Miller et al. 1996, Miller et al. 1999, Roberson 1992). In pollen tubes this has been suggested to be a site where actin is dynamically arranged into microfilaments, the polarized ends of which then impart polarity on the cell (Vidali and Hepler 2001). In oomycete hyphae an F-actin depleted zone has only been reported once (Jackson and Heath 1990) although its existence is a controversial issue as in this study it appeared that not all F-actin was stained. Sub apical regions lacked the brightly stained fibrils and plaques typically seen in fixed hyphae. Jackson and Heath (1990) hypothesized that this may have been due to the low concentrations of rhodamine phalloidin used and/or the blocking of binding sites for the label by actin-binding proteins. It is not known whether these factors led to incomplete staining at the very tip or whether the staining observed indicated the existence of an F-actin depleted zone. This is a critical shortfall in our knowledge as such a structure may well play a critical role in the process of tip growth.

The question of just how closely does what we see resemble the actual situation in a living cell, is the key critique of any study of the cytoskeleton and indeed cell structure. If we are making our observations on live cells then it is generally assumed that we are attaining as close an approximation as possible to the actual distribution of the F-actin. With such cells caution is required in the interpretation of staining patterns as in vivo labeling of actin may stabilize F-actin, may cause over expression artifacts and may block binding sites and thereby compete with actin-binding proteins. Despite these reasons for caution, imaging of live material is the methodology of choice. Due to technical difficulties, however, oomycetes (and indeed most fungi) have proven difficult to load with labels and thus imaging of live cells is, in many cases, not possible. In such circumstances hyphae can be fixed, either chemically or using rapid freeze fixation and observations are made with the

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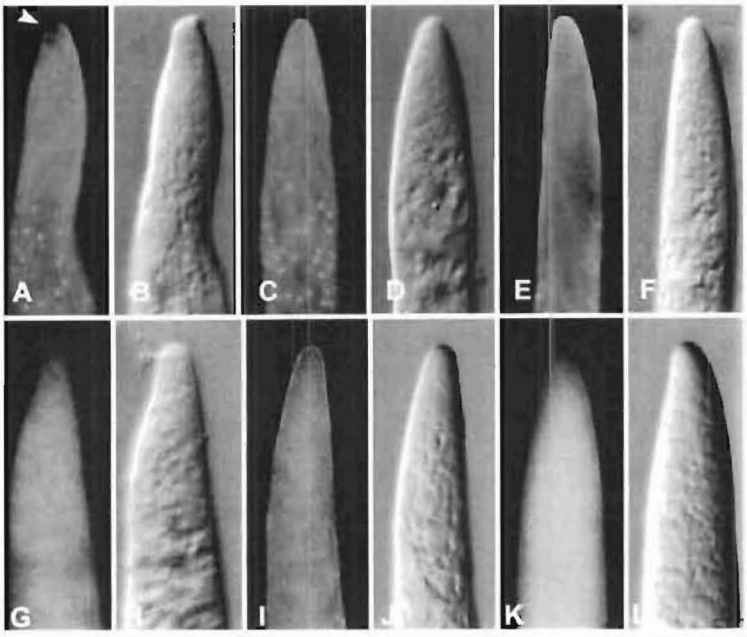

Fig. 1 The effect of various fixatives on the actin-staining pattern and cytoplasm of hyphae labeled with Alexa phalloidin. Fixatives consisted of a combination of 4% formaldehyde and 0.5% methylglyoxal (A, B, C, D), 4% formaldehyde (E, F), 1% methylglyoxal (G, H), 4% glutaraldehyde and 0.5% methylglyoxal (I, J) and 1% glutaraldehyde (K, L). The cytoskeleton in A, C and E is well preserved with an apical cap and subapical plaques. In A an addition structural component, the F-actin depleted zone is evident (arrow). In G there is poor preservation of the cytoskeleton while fine detail is lost in I and K due to poor contrast caused by autofluorescence. DIC images of the cytoplasm (B, D, F, H, J and L) are consistent irrespective of fixative with no evidence of gross cytoplasmic movements. Bar = 5 μm.

assumption that the distribution of F-actin is faithfully preserved by fixation. The validity of this assumption is reliant upon the quality of fixation.

Aldehydes have been widely used as chemical fixatives due to their ability to cross-link proteins (Glauert and Lewis 1998). Of all tested aldehydes, glutaraldehyde is the best cross-linker, as with two functional aldehyde groups, it can rapidly and irreversibly react with a number of amino acids, in particular lysine. This interaction is thought to account for much of its cross-linking (Glauert and Lewis 1998). Its use is problematic, however, when post-fixation labeling involves the use of fluorescent probes such as Alexa phalloidin. Glutaraldehyde-induced fluorescence leads to a high background, which can result in poor contrast. This may mask the fine details of the distribution of F-actin.

An alternative fixative is formaldehyde, which does not auto fluoresce and penetrates tissue more rapidly than glutaraldehyde (Glauert and Lewis 1998). Unfortunately it is not as good a fixative as glutaraldehyde, as with a single functional aldehyde group, it forms fewer and less stable cross-links. Investigators are therefore faced with a situation in which it is possible to get good fixation but poor contrast, or alternatively, sub-optimal fixation but good contrast. The latter alternative is typically preferred with formaldehyde the fixative of choice, although when observations of fine detail are not required the best approach may be a compromise with a fixative comprising low levels of glutaraldehyde in combination with formaldehyde. Regardless of the chosen methodology, the loss of contrast (through the use of glutaraldehyde) and/or structural information (through the use of formaldehyde) may, indeed, mean that our picture of the distribution of F-actin in oomycetes is incomplete.

One aldehyde that has to the best of our knowledge been little used for fixation purposes is methylglyoxal. Like glutaraldehyde it has two functional aldehyde groups, thus it is likely to form many stable cross-links. If it does not cause auto fluorescence, methylglyoxal may give good fixation and enable high-contrast imaging. It is thus important that this is tested as it may impart important information with regard to the actin cytoskeleton and an F-actin depleted zone in oomycetes.

Using hyphae of the oomycete *Achlya bisexualis* we show that fixatives containing a mixture of methylglyoxal and formaldehyde do not cause auto fluorescence and when these are subsequently stained with Alexa phalloidin give higher quality images of the actin network than those fixed with a number of other fixatives. We report the presence in approximately one half of hyphae of F-actin depleted zones in the apical actin cap or an absence of the cap at the very tip. In growing hyphae we report the existence of a cytoplasmic clear zone that may represent the F-actin depleted zone.

Results

All hyphae (n = 227) fixed with a combination of 4% formaldehyde and 0.5% methylglyoxal had an apical cap of F-actin that transformed into peripheral plaques and coarse cables in sub apical regions (Fig. 1A–D; see Fig. 3A–C). A subset of these hyphae (48%) had, within the apical cap, a distinct zone that was deplete of F-actin staining (the F-actin depleted zone) (Fig. 1A; see Fig. 3A, B). The remaining 52% showed no evidence of an F-actin depleted zone (Fig. 1C; see Fig. 3C). This zone was not present in hyphae fixed with any of the other fixatives that we tested (n = 100 hyphae for each fixative). With

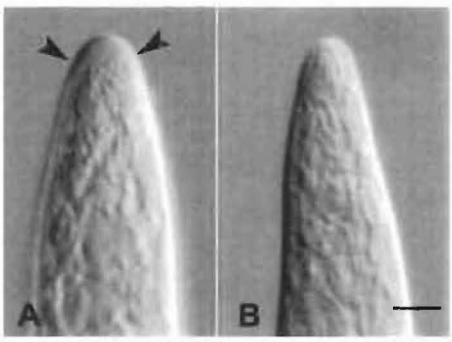

Fig. 2 DIC images of live growing hyphae. Typically two types of cytoplasmic organization were visible at the tip. One showed an apparent clear zone in the cytoplasm (starting at the arrows) where organelles and particles larger than the limit of resolution were not present. The other showed no such zone. Growing hyphae were observed switching between the two organizational types. Bar = 5 μm.

these we typically observed an apical actin cap and plaques and cables in the subapical regions (4% formaldehyde (Fig. 1E; see Fig. 3D)), no apparent structure (1% methylglyoxal (Fig. 1G)), or significant autofluorescence that through reduced contrast masked structural detail (4% formaldehyde and 0.5% glutaraldehyde (Fig. 1I); 1% glutaraldehyde (Fig. 1K)). The respective cytoplasmic organizations at the tips of the fixed hyphae were similar, regardless of the fixative used (Fig. 1B, D, F, H, J, L). There was no discernable difference in the diffraction patterns observed suggesting that there were no detectable artificial gross alterations of cytoplasm at the tips during fixation.

In order to discern whether the appearance of an F-actin depleted zone is due to better preservation of the actin cytoskeleton by the 4% formaldehyde and 0.5% methylglyoxal fixative, relative to the other fixatives, we looked for anything in living growing hyphae that might correlate with such a structure, using DIC optics. When the cytoplasm at the tips of such hyphae was observed, two distinct types of organization were evident. In one of these types there was an apparent clear zone at the very tip where the cytoplasm appeared very smooth (Fig. 2A). The smooth appearance in this zone implies that only organelles and particles that are below the limit of resolution are present. In the other type of organization no such clear zone existed and the cytoplasm had a granulated appearance that was consistent with that seen throughout the hypha (Fig. 2B). Growing hyphae appeared to continually switch between these organizational types, hence the clear zone appeared to be a dynamic structure. We have been unable to observe this clear zone in the cytoplasm of fixed hyphae (see for example Fig. 1B) but its presence may be hidden by the typical reduction in diffraction that occurs with the permeability changes in fixed material.

To further assess the quality of fixation we next examined all fixed hyphae that had been growing at the time of fixation (rather than those that showed the most extensive staining which we have shown in Fig. 1). We restricted this part of the investigation to a comparison of the 4% formaldehyde and 0.5% methylglyoxal and the 4% formaldehyde fixatives as these gave the better actin patterns (as shown in Fig. 1). The combination of methylglyoxal and formaldehyde gave brighter

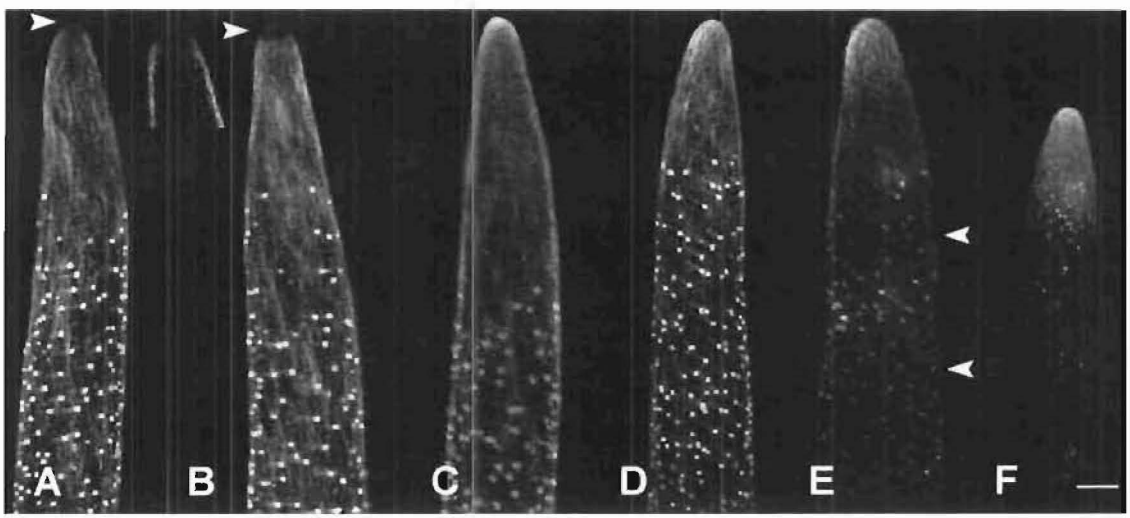

Fig. 3 An actin-depleted zone (arrows) was present at the apex of 48% of hyphae fixed with a combination of 4% formaldehyde and 0.5% methylglyoxal prior to labeling with alexa phallodin (A, B). The inset of 2B shows a single section taken at the median focal plane that displays a clear lack of peripheral staining. Fifty-two percent of hyphae, fixed with the combination, did not show this zone (C). The apical actin-depleted zone was never observed in hyphae that were fixed with formaldehyde alone (D–F). This observation was made regardless of whether the staining of the cells was good (D), moderate (E) or poor (F). In the moderately and poorly stained hyphae, sub apical regions often had areas of incomplete staining, suggesting poor preservation of the actin network (arrows in E and the entire sub apical region in F). Aside from the inset of 2B, all other images are projected Z series. Bar = 5 μm.

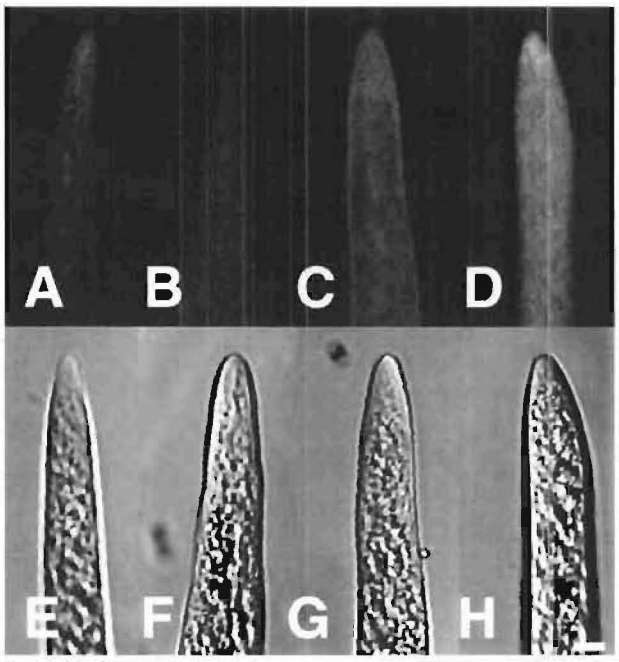

Fig. 4 The effect of the various fixatives on auto fluorescence. The relative amount of auto fluorescence is shown in A–D (which are projected Z series images) with the respective pseudo-DIC images shown in E–H. Hyphae were fixed in either 4% formaldehyde and 0.5% methylglyoxal (A), 1% methylglyoxal (B), 4% formaldehyde and 1% glutaraldehyde (C) or 1% glutaraldehyde (D). A and B show very little auto fluorescence compared to C and D. Bar = 5 μm.

images and stained more of the actin network than the 4% formaldehyde fixative (Fig. 3A–C compared to Fig. 3D–F). Staining was also more consistent as all hyphae showed comparable staining to those shown in Fig. 3A–C. In contrast, hyphae fixed in formaldehyde gave quite variable results, as shown by the varying degrees of staining in Fig. 3D–F in which we present a well-, a moderately and a poorly stained hypha. We have repeated this experiment on five separate occasions and each time have observed these differences. This result implies that more F-actin is preserved and is accessible to phalloidin in cells that have been treated with the combination fixative, possibly due to superior cross linking.

We further investigated the quality of fixation by monitoring organelle movements as hyphae were fixed. Organelles were selected from various positions relative to the tip and hyphal flank and their movements were observed. A Kruskal-Wallace analysis of variance test showed no significant differences between these mean times taken for all movements to stop after addition of fixative ($P > 0.05$). The mean times (in seconds) (± SD) for complete cessation of movements were 56±36 s for 4% formaldehyde and 0.5% methylglyoxal ($n = 6$), 111±40 s for 4% formaldehyde ($n = 8$), 106±132 s for 1% glutaraldehyde ($n = 8$), 157±80 s for 4% formaldehyde and 0.5% glutaraldehyde ($n = 7$) and 153±209 s for 1% methylglyoxal ($n = 5$).

Finally, to determine whether fixation with methylglyoxal causes hyphae to auto fluoresce we used the combination of 4% formaldehyde and 0.5% methylglyoxal as fixative and compared this to fixatives of 1% glutaraldehyde alone, 4% formaldehyde alone and a combination of 0.5% glutaraldehyde and 4% formaldehyde respectively. Very little auto fluorescence was observed in hyphae that had been treated with the 4% formaldehyde and 0.5% methylgloxal fixatives (Fig. 4A) or with formaldehyde alone (Fig. 4B). This contrasted with hyphae that had been treated with fixatives that contained glutaraldehyde. In these hyphae background fluorescence was observed (Fig. 4C, D).

Discussion

Previous studies of the actin distribution in formaldehyde-fixed oomycete hyphae have shown a cap of actin filaments, adjacent to the apical membrane, and a sub-apical network of widely spaced fibrils and plaques (Heath 1987). In this study we show that the incorporation of methylglyoxal in the fixative gives a similar overall actin pattern, but in approximately one half of hyphae, an additional structural component, the F-actin depleted zone is visible. This has previously only been described in live, electroporated hyphae (Jackson and Heath 1990), although there are indications that in that study not all actin may have labeled (see below). Irrespective of this, to the best of our knowledge, this paper represents the first report of an F-actin depleted zone in chemically fixed oomycete hyphae. We suggest, for the reasons given below, that the ability to observe this structure reflects the quality of fixative relative to other aldehyde fixatives.

While an actual quantification of the quality of fixation is difficult, in comparing actin staining patterns of hyphae that were fixed with the combination versus methylglyoxal or formaldehyde alone, we were able to observe the overall F-actin distribution typical of oomycetes more frequently. This suggests that across a sample of hyphae the fixation and subsequent staining is much more consistent with the formaldehyde-methylglyoxal combination. Secondly, with the combined fixative the fluorescence of labeled actin filaments was brighter. This suggests that a higher number of actin filaments remained and were accessible in these cells, following fixation. Thirdly, all of the cells had a more extensively labelled network whereas only 38% had comparable extensive labeling with formaldehyde alone. The remaining 62% of cells had regions of incomplete staining providing further evidence that the cross-linking of the filamentous actin network was better with the combination fixative. Finally, it is worth noting analogous work on pollen tubes. It was initially thought from conventionally fixed cells that there was a dense concentration of actin in the tips (Pierson 1988). More recent work, however, using techniques that are likely to preserve cellular structure better, such as rapid freeze fixation and green fluorescent protein in live cells, suggests that there is limited detectable F-actin in the tips

(Vidali and Hepler 2001, Geitmann and Emons 2000). Whether these F-actin depleted zones are completely free of F-actin is open to debate and they may represent areas of delicate, unstable F-actin, such as those described in pollen tubes (Gibbon et al. 1999) and also in the tips of algal rhizoids (Hable et al. 2003).

The improvement in fixation may be due to structural similarities between methylglyoxal and glutaraldehyde. Both are dialdehydes that primarily cross-link lysine amino acid residues. Glutaraldehyde is thought to be such a good fixative because its two functional aldehyde groups can react rapidly and irreversibly with proteins (Glauert and Lewis 1998). Methylglyoxal is also a dialdehyde and should offer comparable cross-linking to glutaraldehyde without, as our data demonstrates, the problems of auto fluorescence. We suggest that the use of a combination of 0.5% methyglyoxal and 4% formaldehyde may preserve the cytoskeleton of oomycetes better than formaldehyde alone. While not regarded as the best cross-linker, formaldehyde has been widely used as a fixative and, on its own, has revealed an F-actin depleted zone in eufungal hyphae (Roberson 1992). It penetrates tissue rapidly which may be of importance, given the recent suggestion of Foissner et al. (2002) that an organelle-free zone in pollen tubes was better preserved by fast fixation rather than the gradual introduction of fixatives. We suggest that our combination fixative offers fast penetration (formaldehyde) and subsequent irreversible cross-linking (methylglyoxal), without the problems of auto fluorescence that are evident with glutaraldehyde.

Despite an improved methodology for chemically fixing hyphae, it is still unclear as to how closely the actin distributions that we have observed match the actual actin distributions in a living, growing hypha. It should be remembered that our observations have been made on fixed hyphae and therefore do not address the spatial and temporal dynamics of the actin as a hypha extends. A definitive study requires the imaging of all actin at high resolution and sensitivity in a living cell (Heath 2000). This is theoretically possible with the use of GFP-actin or GFP-talin, but in hyphal organisms this approach has yielded limited success (Doyle and Botstein 1996). GFP-talin has been used with tip-growing pollen tubes and interestingly an F-actin depleted zone in the tips was observed (Kost et al. 1998). Alternatively, an actin label can be incorporated into live cells by electroporation or microinjection and these techniques have, like GFP-talin, revealed F-actin depleted zones in pollen tubes (Miller et al. 1996, Anderhag et al. 2000). Unfortunately, reports using electroporation or microinjection on hyphal organisms are limited, presumably due to technical difficulties. In one of the few such reports, an F-actin depleted zone similar to the zone observed in this work was reported, although, in contrast to the current and to other studies, the sub apical peripheral actin network of plaques and cables was not present suggesting that not all F-actin may have labeled with rhodamine phalloidin (Jackson and Heath 1990). It is possible that the electroporation technique or the probe affected the F-actin localization and/or labeling and this may account for the F-actin depleted zone at the tip.

It is tempting to speculate that the clear zone that we observed in the tips of growing hyphae represents the site of the F-actin deplete zone and that it may be analogous to the apical clear zone described in pollen tubes (Lancelle and Hepler 1992). The appearance and disappearance of this clear zone in hyphae is unlikely to be caused by the movement of vacuoles toward and then away from the tip as vacuolar movements of this type typically occur when growth stops and then restarts. It is possible that the lack of a diffraction pattern in this area may be indicative of a lack of F-actin thereby removing any actin-based particle movement. In pollen tubes the clear zone represents an area that is devoid of larger organelles such as mitochondria and Golgi and it has been hypothesized that these are either filtered out by sub-apical fine F-actin (Kost et al. 1998) or undergo stratification on the basis of their size (Vidali and Hepler 2001). Similar organelle distributions have been described in freeze-substituted hyphae of the oomycete *Saprolegnia ferax*, with larger organelles such as mitochondria, Golgi and nuclei largely excluded from the most apical 5 μm, an area that is abundant in wall vesicles (Heath and Kaminskyj 1989).

Perhaps one of the more intriguing questions that this study raises is how an F-actin depleted zone might fit into models of hyphal tip growth. Contrasting models suggest that actin may either resist force at the tip, if growth is powered by turgor pressure, or may provide a protrusive force at the tip, if growth is akin to amoeboid movement in animal cells (Heath 1995). Such protrusive forces in animal cells are thought to arise from the insertional polymerization of actin microfilaments (Pantaloni et al. 2001). There have been suggestions that both turgor-driven and amoeboid models may be correct and that, depending upon environmental conditions, both types of growth may occur concurrently in different hyphae that constitute a single mycelium (Garrill 2000). In both turgor-driven and amoeboid growth models cell wall precursor containing vesicles would need to transported to and be exocytosed at the tip. The F-actin depleted zone may present a means to allow the internal determination (and hence regulation) of an area that would more readily yield to turgor pressure or, alternatively, a protrusive force. It may also enable the exclusion of larger organelles at the tip and would also presumably represent the major site of exocytosis of wall material. With this in mind it is interesting to note the finding of Ketelaar et al. (2003) that a localized instability of the actin network in root hairs can determine the site of exocytosis.

In summary, we present an improved methodology for the chemical fixation of oomycete hyphae that reveals an F-actin depleted zone at the tips of oomycete hyphae. The significance and universality of this F-actin depleted zone will be a critical debate for biologists to resolve in the future if we are to understand the mechanism of tip growth.

Materials and Methods

Hyphae of the oomycete *A. bisexualis* Coker (available from the University of Canterbury culture collection) were grown on cellophane strips overlaying PYG media (Lundy et al. 2001). Prior to fixation hyphae were cut 1 cm behind the growing tip and immobilized in a thin layer of 2% low melting point agarose (Sigma). They were covered with liquid PYG and left for 30 min to allow growth to resume.

For fixation the relevant aldehyde(s) was/were made up (at the relevant concentrations) in 50 mM PIPES (Sigma, St. Louis, MO, U.S.A.) buffer (adjusted to pH 6.8 with KOH (BDH)). Various concentrations (50, 60, 80, and 100 mM respectively) of buffer were tested in preliminary experiments to determine the optimal concentration. Fifty mM was found to give the best preservation and was thus used for all subsequent experiments. Hyphae were fixed with a mixture of 0.5% methylglyoxal (Sigma) and 4% formaldehyde (ProSciTech., Thiringowa Central, Queensland, Australia), 4% formaldehyde, 0.5% glutaraldehyde and 4% formaldehyde, 1% methylglyoxal or 1% glutaraldehyde (Ted Pella Inc, Redding, CA, U.S.A.) for 30 min and then rinsed twice in buffer solution.

Staining of the actin cytoskeleton was accomplished using Alexa Phalloidin (Molecular Probes) according to the method of Heath (1987). The sample was covered with tin foil (to prevent photo bleaching) for 30 min and then washed twice with buffer solution, each time for 15 min. The sample was covered with tin foil between washes. After removal of the second wash the antifading agent *p*-phenylenediamine (Sigma) was added (made up to 0.1% (w/v) in dd H_2O). Samples were examined immediately after staining using an MRC1024 confocal microscope (Bio-Rad, Mississauga, Ontario, Canada) or an Olympus BH-2 Epifluorescent microscope.

We restricted our observations to those hyphae whose morphology and F-actin staining pattern indicated that they had been growing at the time of fixation. This approach is necessary as the structure of the F-actin network differs between growing and non-growing hyphae. Hyphae that had rounded rather than tapered tips and those that did not stain were excluded from the analysis.

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Chapter 3
The effect of RGD-containing peptides on tip growth, F-actin distribution and hyphal ultrastructure

3.1 Introduction

The attachment of metazoan cells to their surroundings is important in determining cell shape and in maintaining proper cell function and tissue integrity. Such binding helps anchor cells and provides positional signals that direct cellular traffic and differentiation (Ruoslahti & Pierschbacher, 1987). As described in the introduction, integrins are integral membrane proteins that exist as heterodimers containing α and β subunits and which contain extracellular, transmembrane, and intracellular segments. They interact with the ECM via their extracellular segments binding a variety of ligands such as ECM proteins and components of the surfaces of other cells (Hynes, 1992). These ligands often contain Arg-Gly-Asp (RGD) sequences. Synthetic peptides containing this RGD sequence can competitively inhibit the binding of integrin to vitronectin or fibronectin of the ECM and have thus proven useful tools in the study of integrins.

Hyphal and yeast morphogenesis depends on the accurate regulation of cell surface extensibility, which involves the membrane skeleton-PM-ECM ensemble (Gupta & Heath, 1997; Heath, 1990; 1994; 1995; Jackson & Heath, 1990a; Kaminskyj & Heath, 1996). Such an ensemble may also be important for developmental processes as actin

appears to have multiple roles in the formation and architecture of zoospores of oomycetes (Heath & Harold, 1992) RGD peptides can also induce the differentiation of germ tubes into appressoria (Correa *et al.*, 1996). Furthermore, there have been suggestions that in the oomycetes, which can grow in the apparent absence of turgor pressure, such proteins may play a role in the provision of a protrusive force at the hyphal apex (Heath & Skalamera, 2001).

In walled cells a number of responses including pertubations of cell wall/plasma membrane adhesion sites, growth, development and differentiation have been observed upon addition of RGD-containing peptides. This has led to the suggestion that proteins analogous to the integrins are present in plants, fungi and oomycetes. The first report was that of Schindler *et al.*, (1989) who reported that RGD sequences significantly enhanced the growth rate of soybean root cells, and affected cell wall and plasma membrane interactions and organisation. Subsequently they were shown to prevent adhesion-dependent gravisensing in *Chara* (Wayne *et al.*, 1992). In sunflower protoplasts Barthou *et al.*, (1999) found that an RGD containing heptapeptide reduced sunflower embryoid formation by as much as 50%. The authors suggested that disruption involved cortical microtubules that were linked to RGD-binding proteins that bound to components of the agarose matrix that the protoplasts were embedded in. Involvement of actin microfilaments and RGD-sensitive cell wall plasma membrane interactions in the process of cytoplasmic streaming in mesophyll cells of *Vallisneria* was suggested by the work of Ryu and coworkers (Ryu *et al.*, 1997). Addition of RGD- (and also RYD-) containing peptides was found to induce extremely abnormal patterns of cytoplasmic streaming. Disruption of wall membrane interactions has been reported in a number of other species including *Arabidopsis thaliana* (Canut *et al.*, 1998), *Chara* (Katembe *et al.*, 1997), maize (Laboure *et al.*, 1999) and pea (Kiba *et al.*, 1998).

With respect to tip growth there have been a number of relevant studies although, with respect to roles for RGD sensitive molecules the results are at times contradictory. Pereyra *et al.*, (2003) found that addition of the tripeptide RGD to the culture media of the fungus *Mucor rouxii* delayed the switch from isodiametric growth to tip growth. This suggests that RGD can affect the establishment of cell polarity, a fundamental requirement for tip growth. In addition, the peptides reduced the number of plasma

membrane cell wall adhesion sites (which are discussed more fully in chapter 4) in plasmolysed *Pelvetia* rhizoids (Henry *et al.*, 1996), but contrary to this they had no apparent effect on hyphae of the oomycete *Saprolegnia ferax* (Bachewich & Heath, 1997a). Furthermore, oomycete hyphae (Kaminskyj & Heath, 1995) and fungal germlings (Correa *et al.*, 1996) are able to grow in RGD-containing media. It should be noted however that in this latter study the germlings were initially swollen and were unable to form appressoria. Pollen tubes have been reported to slow their growth in response to RGD-containing peptides but, as detailed in the Discussion of this chapter there are technical problems with this work that were not recognised by the authors (Sun *et al.*, 2000).

The contradictory nature of some of the observations on tip growing cells suggest this is an area in which further work is required particularly in view of additional lines of evidence that are described below that suggest the presence of integrin-like proteins in tip growing cells. Hyphae and pollen tubes display cross-reactivity to antibodies raised against the β1-subunit of animal integrins (Kaminskyj & Heath, 1995; Sun *et al.*, 2000) and integrin associated proteins such as spectrin and the vitronectin receptor (Degousee *et al.*, 2000; Kaminskyj & Heath, 1995; Sun *et al.*, 2000). Significantly, these molecules appear to be localised to the apex where many of the processes characteristic of tip growth occur. Immunoblots of hyphal fractions have identified a 178 kDa protein with β1 integrin antibody affinity. Under reducing conditions this converted to a 120 kDa band which is of a similar size to animal integrins (Kaminskyj & Heath, 1995). Correa *et al.*, (1996) used an RGDSPC affinity column to isolate at least twelve proteins from *Uromyces appendiculatus* germlings, one of which, a 95 kDa protein displays reactivity with two different β1 antibodies. In contrast, membrane fractions from pollen tubes show no β1 integrin cross reactivity, instead a protein of approximately 150 kDa has been isolated that reacts with β3 and α5 integrin subunit antibodies. Integrin-like proteins do not appear to be restricted to tip growing cells as antibody staining has been reported in a number of other walled cell types and in fractions derived from these cells (de Ruijter & Emons, 1993; Gens *et al.*, 1996; Lynch *et al.*, 1998; Quatrano *et al.*, 1991; Reuzeau & Pont-Lezica, 1995; Sanders *et al.*, 1991; Swatzell *et al.*, 1999; Wagner *et al.*, 1992).

There have been reports of genes that encode integrin-like products in walled cells (Gale *et al.*, 1996; Hostetter, 1999; Nagpal & Quatrano, 1999). One of these, called INT1, when disrupted in *Candida albicans*, suppresses hyphal growth. It should be noted however that sequence similarity between this and animal integrins is at best very low and most authors conclude that, on the basis of the non-metazoan genomes published thus far that integrins and true integrin homologues are likely restricted to the metazoa. It is however also worth noting the remarks of Wasteneys & Galway, (2003) who caution the use of genomic databases to discount conclusively the existence of specific proteins. Regardless of this, immunological data suggest that in walled cells proteins do exist with sufficient epitopic similarity to animal integrins to allow cross reactivity. Furthermore, the response to RGD-containing peptides suggests that proteins with functionally analogous roles to those of the animal integrins are likely to exist in plants, fungi and oomycetes although the role that these may (or indeed may not play) in the process of tip growth and differentiation are as yet unknown. This chapter attempts to address this shortfall in our knowledge.

3.2 Material and methods

3.2.1 Culture

Stock cultures of *Achyla bisexualis* were maintained on the complete nutrient medium PYG (Hintz & Horgen, 1983). For experimental purposes, hyphal mats were grown on strips of cellophane overlaying PYG medium (2% w/v agar). After cutting hyphae approximately 1 cm behind their apices, they were allowed a 1-h recovery period. The hyphae were then mounted in slide chambers and embedded in 3% low-melting-point agarose (Type VII; Sigma Chem. Co., St. Louis, MO), and submerged with liquid PYG. The slide chamber was made by fixing a coverslip over a hole that had been drilled through a glass microscope slide (for more details see Chapter 5).

3.2.2 The effect of RGD-containing peptides on hyphal growth rate

In order to test the effects of RGD-containing peptides on rates of hyphal tip growth the peptides RGD and RGDS (Sigma, St. Louis, MO) were used. After setting up a slide chamber, as described in Section 3.2.1, and allowing a 1-hour recovery period, hyphal

extension was measured, before, during and after exposed to 0.2 ml liquid PYG containing the relevant concentration of RGD or RGDS. These solutions were added and subsequenctly removed by gentle profusion. Growth rate were also monitered in the presence of the tripeptide GGR (in which the sequence RGD is not present), in potassium acetate and in SES (standard extracellular solution; containing 10 mM KCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM HEPES adjusted to pH 7.0 with 2.5 mM KOH). For controls used in subsequent experiments, growth rates in the presence of tetrapentyl ammonium chloride (TPA) were also measured.

Growth rates were measured using a Confocal Laser Scanning Microscope (MRC 1024; Bio-Rad, Mississauga, Ont.) via an Olympus IX 70 (inverted) microscope with a 1.4 NA, 20x or 40x objective.

3.2.3 The effect of RGDS on actin organization

To determine the effect of RGDS on the F-actin cytoskeleton hyphae were prepared as described above and then exposed to 1.5 mM or 3 mM RGDS for either 30 sec or 30 min. For controls hyphae were exposed to either 0.25 mM TPA, 3 mM GGR, SES or 3 mM potassium acetate. After exposure to the relevant treatment hyphae were fixed, stained and observed as described in Chapter 2. In an attempt to quantify the effects of the treatments on F-actin staining patterns F-actin intensity profiles were created using Metamorph software version1.0. First, Z-series images of actin staining of an individual hypha were converted to a 3D projection image. The image was then calibrated using a micrometer scale on the slide of the image. The F-actin intensity profiles of the 3D projection image was then created using the MetaMorph linescan function in which the intensity was measured and the data logged. From the image's pixel intensity value, the lowest value was set as 0 and the highest value as 1. Nine hyphae were averaged for each treatment and the relative intensity versus distance from the tip was plotted. No attempt was made to separate those hyphae which had F-actin depleted zones from those which did not.

3.2.4 The effect of RGDS on hyphal ultrastructure

To determine the effect of RGDS on hyphal ultrastructure hyphae were prepared as described above and were then exposed to 1.5 mM RGDS or 0.25 mM TPA for 30 min

before they were fixed and stained. For transmission electron microscopy, hyphae were fixed in 2.5% glutaraldehyde in 0.075 M phosphate buffer pH 7.2-7.4 for 1 hour, then washed in phosphate buffer 3 times at 10 min intervals. Hyphal mats were then embedded in 3% low melting point agar. After fixation hyphae were exposed to 1% OsO_4 for 1 hour then washed in phosphate buffer three times for 10 min each, dehydrated in an acetone series and infiltrated and embedded in Spurr's resin. Sections were cut using an ultramicrotome (2128 Ultrotome system; LKB, Bromma), stained with 1% uranyl acetate and saturated lead citrate, and viewed with a JOEL 1200EX transmission electron microscope.

3.3 Results

3.3.1 The effect of RGD or RGDS-containing peptides on tip growth

The effects of RGDS, RGD and GGR on the rate of hyphal extension of *Achlya bisexualis* are shown in Table 3.1. RGDS was found to significantly slow hyphal extension ($P<0.05$) relative to hyphae that were exposed to control solutions. These controls were liquid PYG (described as the control hypha in Table 3.1) and the non-RGD containing peptide GGR respectively. At the concentrations tested, the peptide RGD had no significant effect ($P>0.05$) on extension rates relative to the controls. The inhibitory effects of RGDS were found to be dose dependant with the maximal concentration tested of 3 mM slowing growth to around 10% of that prior to addition of the peptide. This is shown graphically in the normalised data that is presented in Figure 3.1. The Figure also shows the lack of effect of additional controls SES and potassium acetate on growth rates. Reasons for the inclusion of these controls are given in the Discussion. The K^+ channel blocker tetrapentyl ammonium chloride at a concentration of 0.25 mM was shown to slow growth to approximately the same degree as 1.5 mM RGDS. TPA was tested, as it was necessary as a control for subsequent experiments on the effects of RGDS on actin distribution (see below) (Figure 3.1).

Growing hyphae of *A. bisexualis* typically have a long taper towards their tips (Figure 3.2). RGDS at 1.5 and 3 mM was found to reduce this giving a more rounded tip with an increasing level of vacuolation in more apical regions (Figure 3.3 and Figure 3.4 respectively). This is particularly so in the hypha in Figure 3.4 in which tip growth has

stopped. Despite the rounding of tips there was no change in hyphal diameter in more subapical regions with either concentration of RGDS. TPA at 0.25 mM had a similar effect with respect to rounding of the tip but in contrast to RGDS there was no apparent increase in vacuolation towards the tip (Figure 3.5). Furthermore TPA increased the diameter of hyphae. These effects are summarised in Table 3.2.

3.3.2 The effect of RGDS on actin organization

RGDS, at a concentration of 1.5 and 3 mM was found to affect the organisation of F-actin. Thus hyphae that had been exposed to RGDS prior to chemical fixation and staining with Alexa Phalloidin showed a much less obvious F-actin cap relative to the control hyphae (Figure 3.6). No such reduction was observed in hyphae that had been treated with TPA prior to fixation and staining (Figure 3.7). In an attempt to quantify these effects fluorescence intensity profiles were created for each of these treatments. Such profiles for control hyphae show a pronounced peak in relative intensity at the tip of the hyphae that gradually dissipates to a basal level approximately 20 μm back from the tip (Figure 3.6D; Figure 3.7C). The profiles for RGDS treated hyphae at both 1.5 and 3 mM show much lower intensities at the tip with a subsequent less steep decrease to the basal level around 20 μm back from the tip (Figure 3.6D) In contrast the fluorescence intensity profile for hyphae exposed to TPA was similar to that of the control (Figure 3.7C). In addition to changes in the F-actin cap the transition from the cap region to the subapical region of plaques was much more immediate in the presence of RGDS compared to the control or TPA treated hyphae. Thus in Figure 3.6A and Figure 3.7A, B there is a gradual appearance of plaques in subapical regions. In contrast this transition is much less gradual in Figure 3.6B, C with an apparent line, perpendicular to the plane of growth of the hyphae marking the transition.

3.3.3 The effect of RGDS on hyphal ultrastructure

Control hyphae were found to contain an abundance of vesicles at the extreme tips of hyphae and, at a lower density, along the flank of the subapical region (Figure 3.8). These hyphae showed the characteristic taper described above. Immediately subapical to the vesicles were mitochondria, which typically orientated parallel to the long axis of the hypha (Figure 3.8A). What are assumed to be small vacuoles were also present as

clear staining organelles that were interspersed with the mitochondria. Hyphae that had been exposed to 3 mM RGDS for 30 minutes prior to fixation, had a more rounded tip, as was expected from the LM observations. Vesicles were less abundant at the tip and also at the flank of subapical regions (Figure 3.8B). Mitochondria moved forward to the tip and again these were present with clear organelles that are assumed to be vacuoles, this is consistant with the LM image in Figure 3.5. Treatment of hyphae for the same period of time with 0.25 mM TPA gave hyphae with rounded tips and enlarged mitochondria (Figure 3.8C). Vesicle distribution and density was similar to that of the RGDS treated hypha. The degree of vacuolation was less in the TPA treated hypha an observation that is consistent with the LM observations. There was also some evidence of nuclei towards the tip.

3.4 Discussion

This chapter shows that the peptide RGDS slows tip growth, affects that F-actin distribution and affects hyphal morphology and ultrastructure. This is consistent with the presence of integrin-like proteins in *A. bisexualis* and a role for these in the tip growth process. While RGDS was found to be effective against tip growth, RGD had no effect. Reports from the animal literature suggest that different cell types show differing sensitivities to various RGD containing peptides, thus some show greater sensitivity to RGDS compared to RGD, while in others the reverse is the case (Ruoslahti, 1996).

The presence of integrins in non metazoan cells is an area that is, largely on the basis of analyses of sequence databases, fraught with controversy. Indeed several authors state quite categorically that integrins do not exist in plants, fungi or oomycetes (Baluska *et al.*, 2003). This is based on the finding that none of the plant, fungal or oomycete sequence databases that have been published contain homologues of integrin genes (Baluska *et al.*, 2003). While the presence of true integrins is certainly open to debate there can be little doubt in these organisms that, firstly there are proteins that have epitopic similarity to integrins (as evidenced by the large numbers of immunocytochemical studies in which oomycetes fungi and plant show cross reactivity to antisera raised against various subunits of animal integrins (de Ruijter & Emons, 1993; Gens *et al.*, 1996; Lynch *et al.*, 1998; Quatrano *et al.*, 1991; Reuzeau & Pont-

Lezica, 1995; Sanders *et al.*, 1991; Swatzell *et al.*, 1999; Wagner *et al.*, 1992) and secondly that protein interactions occur that involve recognition of an RGD containing motif. These data are consistent with the existence of integrin-like proteins.

The data presented contrasts with earlier findings with the oomycete *Saprolegnia ferax* in which hyphae that were exposed to 1.5 mM RGDS grew at normal rates (Kaminskyj & Heath, 1995; Bachewich & Heath, 1997a). It is unlikely that the process of tip growth differs greatly between *S. ferax* and *A. bisexualis* so there may be methodological differences that account for the contrasting data. In the present study the RGD-containing peptides were made up in SES solution, which contains divalent cations. In animals integrins require divalent cations for effective binding of components of the ECM, these cations interact with a motif called the metal ion-dependent adhesion site (MIDAS) that is located within the I domain. Cation binding is thought to promote a conformational change within the domain that enables subsequent interaction with the ECM (Hynes, 2002). One possible explanation for the contrasting data with oomycetes is that the presence of divalent cations in the present study promoted interaction between integrin-like proteins and RGDS. Certainly the lack of effect of RGD-containing peptides on tip growth in *S. ferax* is surprising given the presence of proteins with epitopic similarity to integrins in this organism and the finding that these proteins locate at the tips of hyphae where many of the processes that are critical to tip growth occur.

With respect to other hyphal organisms Correa *et al.*, (1996) have suggested that germlings of the fungus *Uromyces* appendiculatus are able to grow in RGD-containing media although there was an effect on morphology and later developmental process. Furthermore, as in the study of Kaminskyj & Heath (1995), there are proteins in this organism that have epitopic similarity to animal integrins (Correa *et al.*, 1996). Additional effects on development have been reported in the fungus *Mucor rouxii*, in which RGD has been shown to delay the switch from isodiametric growth to tip growth and lead to the formation of more germ tubes (Pereyra *et al.*, 2003).

There have been reports of RGD slowing pollen tube tip growth in "quasi" *in vivo* culture conditions but not in vitro (Sun *et al.*, 2000). These cells also contain proteins that are antigenically related to animal integrins. However, the growth studies should

treated with some caution as they were carried out using Brewbaker and Kwack (BK) medium (pH 6.0) which is unbuffered (Brewbaker & Kwack, 1963). Commercial preparations of RGD containing peptides typically contain large amounts of acetate (see Correa *et al.*, (1996)) and thus there is the possibility that the observed effects may be due to alternations of pH. For this reason it is of fundamental importance that appropriate controls are carried out involving the addition of acetate at the relevant concentration. The fact that acetate had no effect on tip growth rates in the present study suggests that the slowing of growth with RGDS was due to the peptide and not any contaminant in the preparation.

RGD-containing peptides, in addition to studies on growth and development, have also been used in studies relating to fungal adhesion. Such studies suggest the existence of protein receptors in human pathogenic species such as *Candida albicans* and *Aspergillus fumigatus* and in non-human pathogenic species such as *Uromyces appendiculatus* that are able to recognise proteins of a host's extracellular matrix. They are thus likely to contain an RGD-binding motif. The proteins that they interact with in the matrix are thought to include fibronectin, laminin and iC3b. The data presented in the current chapter are consistent with similar receptors also playing a role in tip growth although in this case they will bind to components of their own cell wall. In addition fungal adhesion proteins such as Int1p from *Candida albicans*, the developmentally regulated protein MFBA from the mushroom *Lentinus edodes* and SRAP32 (one of the symbiosis regulated acid polypeptides) from the eucalyptus symbiote *Pisolithus tinctorius* all contain the sequence RGD. It has been suggested that this enables recognition by these organisms of integrins in the host cell that may then facilitate adhesion (Hostetter, 1999).

As detailed in Chapter 1 a possible role for integrin-like proteins in oomycetes is anchorage of the F-actin cytoskeleton. This may then enable F-actin to either resist turgor pressure or to provide a protrusive force at the tip of hyphae. If this is the case then one could predict that RGDS might perturb the F-actin cytoskeleton. Fixation and staining of hyphae that had been exposed to RGDS showed that this is indeed the case. These hyphae showed less intense staining of the F-actin cap, which is suggestive of decrease in the amount of F-actin in this region. It is also possible that the F-actin

concentration remains the same but that it is less able to bind the Alexa Phalloidin in the presence of RGDS. The fact that the basal level was the same in RGDS treated hyphae and the control would argue against this latter possibility. In addition to changes to the F-actin cap RGDS was found to shorten the transition zone between the cap and subapical plaques, making this more immediate.

In the oomycetes changes to the F-actin cytoskeleton are typically associated with changing growth rates. There is thus the possibility that the changes that were observed with RGDS were actually due to the slowing of growth that this peptide caused rather than its direct effect on the cytoskeleton. To address this problem the effect of TPA on the F-actin cytoskeleton at a concentration (0.25 mM) that slowed growth to a similar rate to that observed in the presence of 1.5 mM RGDS was investigated. TPA is an inhibitor of K^+ ion channels that has previously been used to slow tip growth in the oomycetes (Kaminskyj *et al.*, 1992a). The fact that TPA affects the F-actin cytoskeleton in a manner that is quite different to that affected by RGDS is consistent with the changes that are observed with the latter not simply being due to changes in growth rate.

If the role of the actin cytoskeleton were to resist turgor pressure then it might be argued that any decrease in F-actin towards the tip, as has been observed after RGDS treatment, would lead to an increase in the rate of tip growth. Clearly this is not the case as a reduction in growth rate was observed. One possible explanation for this anomaly may be a compensatory strengthening of the cell wall as the turgor resisting capabilities of the F-actin cap are compromised. An additional role of the F-actin cytoskeleton in tip growth is the delivery of vesicle towards the tip. The low concentration of vesicle observed at the tip after RGDS treatment suggests that vesicle delivery is indeed compromised in the presence of RGDS. The delivery of fewer vesicles to the tip with the consequence of a decrease in exocytosis would certainly account for the slower growth rates.

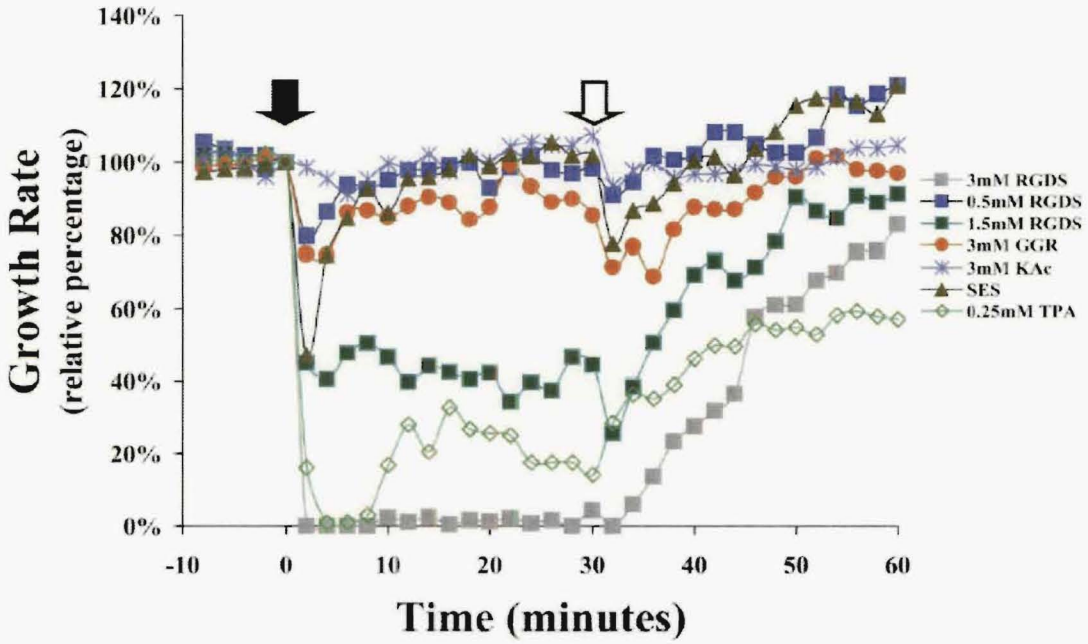


Figure 3.1 The effect of RGDS on the growth rate of *A. bisexualis*. Data have been normalized.

GGR, KAc, SES and TPA (see text for reasons for their inclusion in this assay) were added as controls, the filled arrow represents inhibitor addition and the open arrow inhibitor washout.

RGDS: Arginine-Glycine-Aspartic acid-Serine

GGR: Glycine-Glycine-Arginine

TPA: Tetrapentylammonium Chloride

KAc: Potassium Acetate

SES: Standard Extracellular Solution

Table 3.1 The effect of RGD-containing peptides on rates of tip growth

Growth rates of hyphae				
	Growth rate (μm/min)			
Treatment	Mean	Standard deviation	Range	Sample size
A) Control (PYG broth)	5.67	1.12	3.82-7.07	7
B) 0.5 mM RGDS	3.61	1.66	1.38-5.80	7
C) 0.5 mM RGD	5.13	1.31	3.37-6.80	7
D) 0.5 mM GGR	6.08	0.82	4.99-7.59	7

Based on ANOVA and Tukey test: A≠B, D≠B.

RGD: Arginine-Glycine-Aspartic acid

RGDS: Arginine-Glycine-Aspartic acid-Serine

GGR: Glycine-Glycine-Arginine

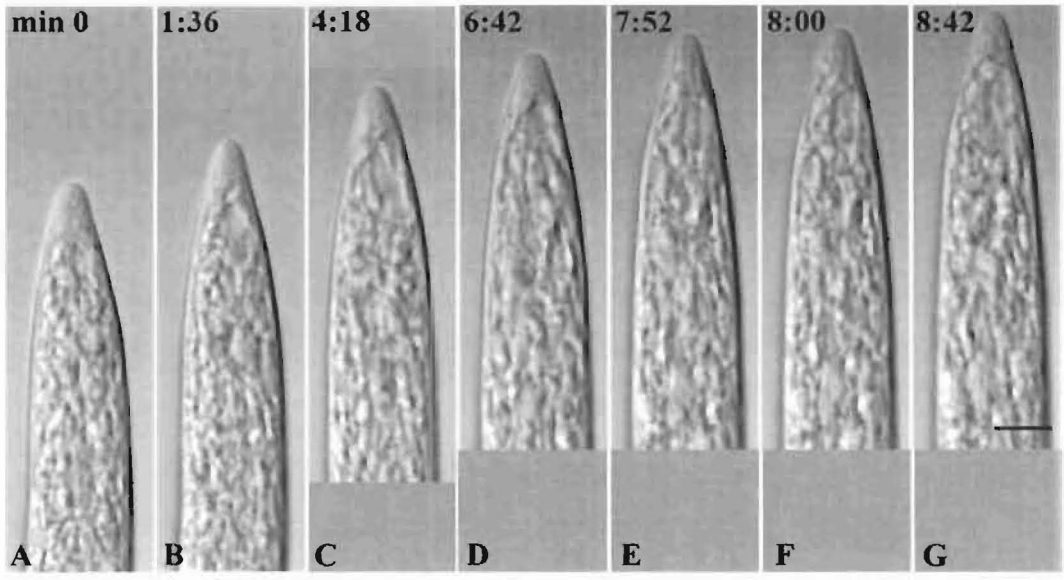


Figure 3.2 Normal growth of *Achlya*

The tip of the hypha shows the typical long tapered shape and the extreme tip is mostly devoid of detectable large organelles. Time is given in minutes. Bar =10 μm

Table 3.2 Summary of the effects of RGD-containing peptides and TPA on hyphal growth of *A. bisexualis*

Treatment / Effect	Control	0.5 mM RGDS	1.5 mM RGDS	3 mM RGDS	0.25 mM TPA
Growth rate	Normal	Normal	Slowed	Slowed or cessation	Slowed
Tip morphology	Tapered	Tapered	Flat	Flat	Flat
Diameter after regrowth	Normal	Normal	Normal	Reduced	Increase*
Is the tapered tip restored after treatment washout	-	Yes	Yes	Yes	Yes

*: The diameter was enlarged after longer treatment

-: Not determined or tip always tapered in control

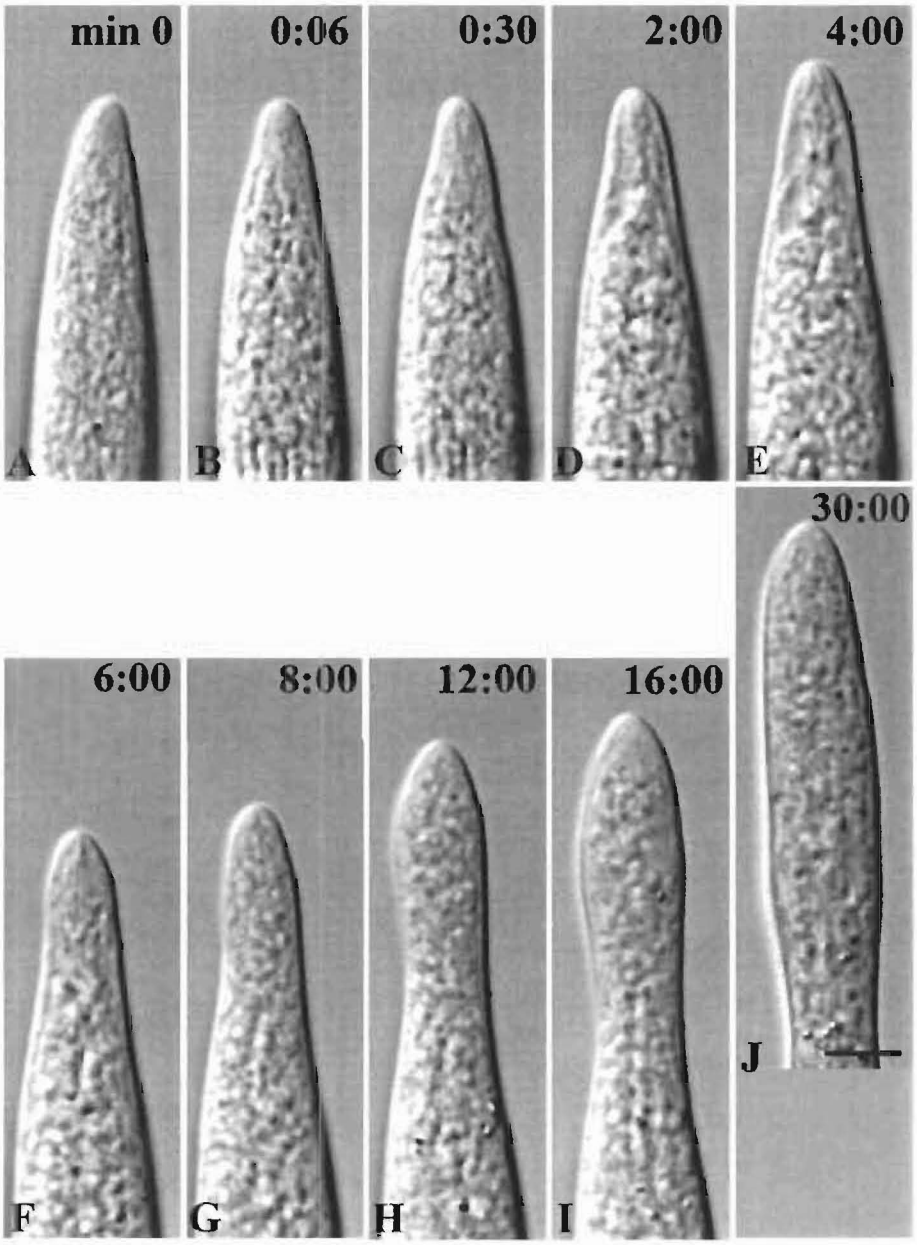


Figure 3.3 The effect of 1.5 mM RGDS on the growth and morphology of hyphae of *A. bisexualis*

In the example shown the time scale represents the time after the addition of 1.5 mM RGDS which slowed the growth of hyphae and affected the shape of the growing tip reducing the characteristic taper (seen prior to addition in A), giving a more rounded tip (B-J). Increasing levels of vacuolation were seen towards the tip after addition of RGDS. Bar =10 μm.

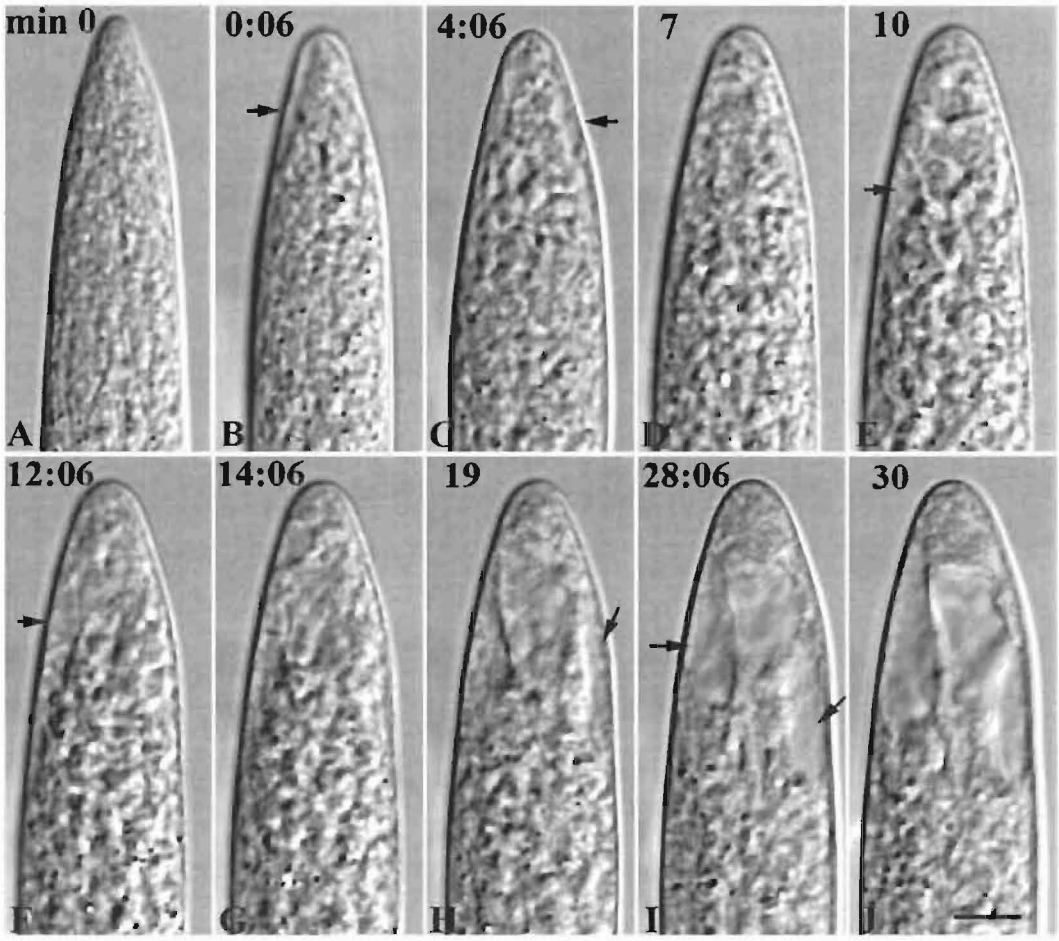


Figure 3.4 The effect of 3 mM RGDS on the growth and morphology of hypha of *Achlya*

In the example shown the time scale represents the time after the addition of 3 mM RGDS which slowed or stopped the growth of hyphae and affected the shape of the growing tip reducing the characteristic taper (seen prior to addition in A), giving a rounded tip (B-J). Increasing levels of vacuolation were seen towards the tip after addition of RGDS (indicated by the arrows). Bar =10 μm

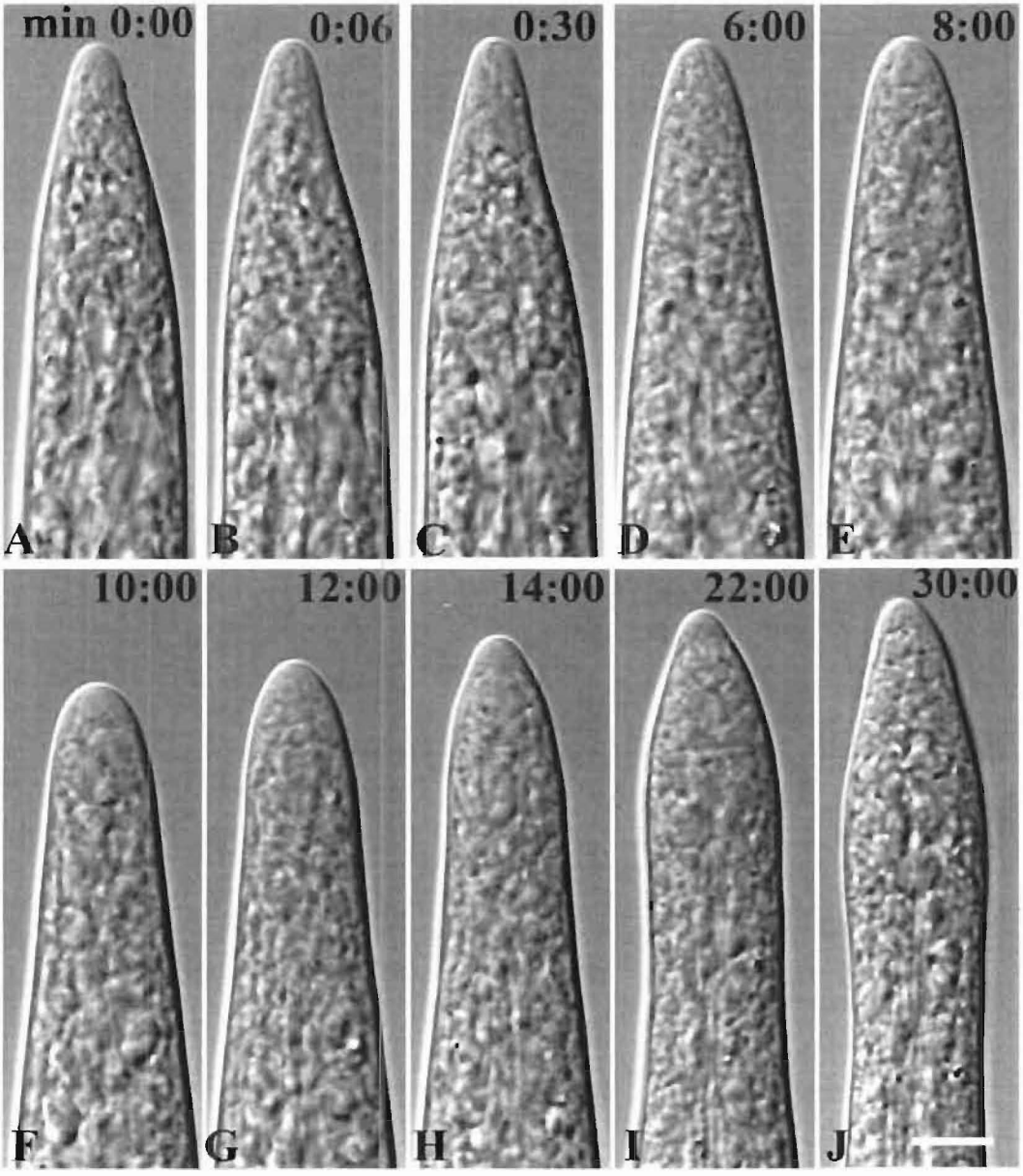


Figure 3.5 The effect of 0.25 mM TPA on the growth of hypha of *Achlya*

In the example shown treatment with TPA caused the tip of hypha to become rounded and slowed the grow rate. There was little evidence for a significant increase in the level of vacuolation at the tip. Bar =10 μm

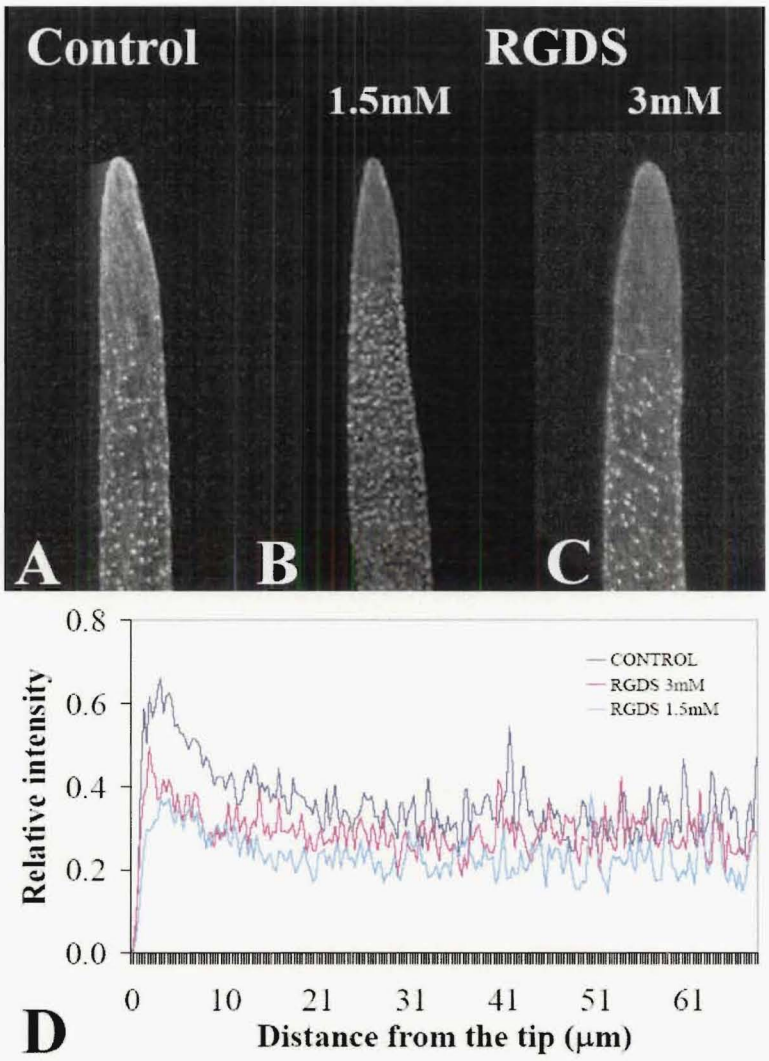


Figure 3.6 An F-actin fluorescence intensity profile for in control hyphae and RGDS treatment.

Hyphae were treated with 1.5 or 3 mM RGDS, then fixed with 4% formaldehyde and 0.5% methylglyoxal and stained with Alexa-phalloidin (B, C). A control hypha that had no prior exposure to RGDS is shown in (A) RGDS was found to reduce the intensity of fluorescence at the tip suggestive of a reduction in the amount of F-actin there (as shown by the reduction in relative intensity in D). It also shortened the transition zone from the F-actin cap to plaques.

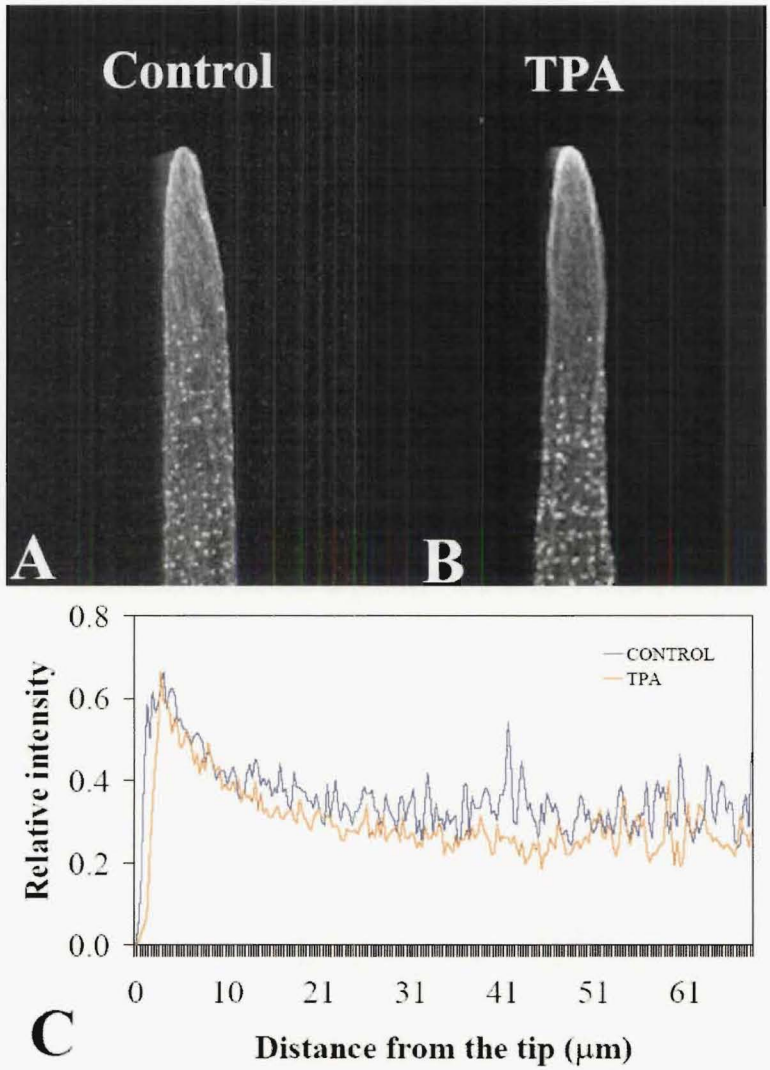


Figure 3.7 An F-actin fluorescence intensity profile for hyphae in control and after TPA treatment.

The hyphae were treated with 0.25 mM TPA, then fixed and stained with Alexa-phalloidin. Hyphae treated with TPA (B) had similar F-actin staining patterns to control hyphae that were not exposed to TPA (A). This is further supported by the plot of the relative fluorescent intensity (C).

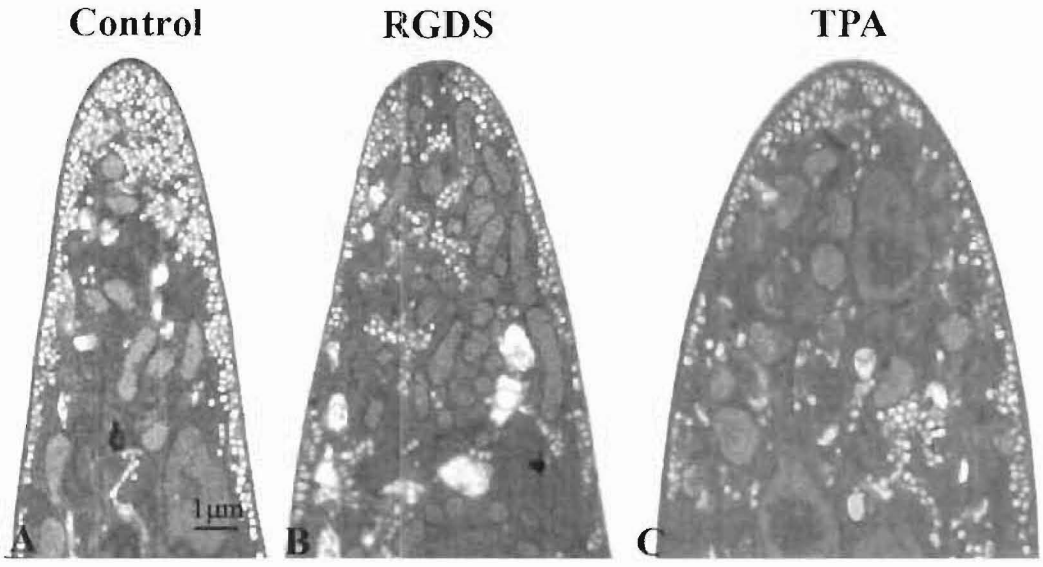


Figure 3.8 Hyphal ultrastructure after treatment 30 min.

Hyphae were exposed to 3 mM RGDS or 0.25 mM TPA for 30min prior to fixation, embedding and sectioning. RGDS and TPA cause rounding of the tip and movement of mitochondria to the tip. There is also a reduction in the number of vesicles at the tip relative to a control hypha. With RGDS there are also vacuoles present.

Chapter 4
The effect of RGDS on cell wall-plasma membrane attachment sites

4.1 Introduction

Plasmolysis is a response of many types of walled cell to osmotic shock. Upon challenge by hyperosmotic media the osmotic loss of water from the cytoplasm and vacuoles causes a reduction in the volume of the protoplasm and the retraction of the plasma membrane from the cell wall. This retraction of the protoplasmic envelope from the cell wall was first described by Braun (1852 as cited by Stadelmann, 1966) although it was apparently De Vries (1877 as cited by Stadelmann, 1966) who first coined the term "plasmolysis" and defined it as the separation of the living protoplasmic envelope from the cell wall, caused by the action of an external water-withdrawing solution (Stadelmann, 1966).

Since its first description plasmolysis has been used extensively as a means of studying, or measuring, a variety of physiological processes (for a review see Oparka *et al.*, 1994). In addition, plasmolysis is the starting point of experimental protoplast isolation and thus plays a fundamental role in ion channel studies (Garrill *et al.*, 1992; Roberts *et al.*, 1997; Very & Davies, 1998). Patterns of plasmolysis are indicative of the physiological state of the protoplast (Stadelmann, 1966) and also, with particular regard to this thesis, of the relationship between the cytoskeleton the plasma membrane and cell wall (Bachewich & Heath, 1997b; Lee-Stadelmann *et al.*, 1984; Oparka *et al.*, 1994).

Many factors can influence the pattern of plasmolysis, these include the type of cells under investigation, as well as the nature of the plasmolyticum which is used. Despite this there are two predominant patterns of plasmolysis that are observed. These are termed the concave and convex forms. In concave plasmolysis, concave pockets form along longitudinal walls as the plasma membrane (PM) separates from the wall at each pocket. In convex plasmolysis the protoplast separates from the transverse walls of each cell forming a symmetrical protoplast with convex ends. Concave plasmolysis typically indicates either relatively high protoplasmic viscosity, or strong PM-wall binding. The convex form indicates relatively lower protoplasmic viscosity or weaker PM-wall adhesion (Stadelmann *et al.*, 1984). The pattern of plasmolysis is typically relatively consistent for any tissue of a given age, but may change during maturation of the tissue or following wounding (Hanchey & Wheeler, 1969; Lee-Stadelmann *et al.*, 1984). When cells are treated with excessively high concentrations of plasmolyticum, a retracted protoplasm or protoplast may fragment into two or more sub-protoplasts (or partial protoplasts) (Attree & Sheffield, 1985; Lee-Stadelmann & Stadelmann, 1989). A subprotoplast may form immediately on addition of the plasmolyticum or develop later from severe concave plasmolysis. The subprotoplasts are frequently joined by PM-bounded protoplasmic strands, or may be attached to the wall by similar PM-bounded strands.

In plasmolysing cells, the presence of adhesions between the plasma membrane and cell walls has been used to suggest the presence of structures that are similar to focal adhesions in animal cells. These structures in animal cells are sites where integrins connect the extracellular matrix to the cytoplasm by way of the cytoskeleton. The bidirectional signalling properties of the integrins make these structures the location of both inside out and outside in signalling processes that are fundamental to cell growth and development. In numerous studies on oomycetes, fungi, and plants adhesions have been observed where masses of cytoplasm and plasma membrane remain attached to the cell walls. These include species and cell types as diverse as fern protonemal cells (Kagawa *et al.*, 1992), tips of oomycete hyphae (Kaminskyj & Heath, 1995), algal rhizoids (Henry *et al.*, 1996), onion epidermal cells (Oparka *et al.*, 1994, Lang-Pauluzzi, 2000), barley plants (Lee-Stadelmann *et al.*, 1984), and *Pteridium* protoplasts (Attree & Sheffield, 1985).

With regard to hyphal organisms and a role for the structural elements that underlie these points of adhesion in tip growth Heath (2001) has suggested that a number of observations are pertinent that are listed below. These are suggestive of a greater abundance and greater strength of adhesions in the tip where most of the processes critical to tip growth occur. Firstly remnants of plasma membrane and actin remain attached to the apical wall upon plasmolysis and are larger here than fine subapical attachment points that resemble Hechtian strands from higher plants (Bachewich & Heath, 1997b; Kaminskyj & Heath, 1995). Secondly, as hyphae plasmolyse the cell wall is drawn inwards in a region immediately behind the tip. At this point the plasma membrane stays attached to the wall via broad adhesions (Bachewich & Heath, 1997b). Thirdly, anti-integrin antibody staining occurs predominantly at the tip (Degousee *et al.*, 2000; Kaminskyj & Heath, 1995). Fourthly, cytoplasmic contractions that occur when hyphae are fixed with formaldehyde occur predominantly toward the tip, indicating attachment at the tip (Heath, 1990). Fifthly, there have been suggestions that peripheral plaques of actin that are observed in the tips of most eufungi are focal adhesion equivalents (Hoch & Staples, 1983; Adams & Pringle, 1984; Roberson, 1992). There is controversy surrounding such suggestions largely because they appear to be mobile in the yeast *Saccharomyces cerevisiae* (Doyle & Botstein, 1996) although whether these represent the same structural elements as that observed in hyphae is open to debate. Finally, as described in the previous chapter fungi can be sensitive to RGD containing peptides, indicative of structures that contain components that are similar to the integrins and the ECM of the metazoa.

In view of the above and the observations presented in Chapter 3 this chapter investigates the effect of RGD-containing peptides on plasma membrane cell wall attachments in *A. bisexualis*.

4.2 Material and Methods

4.2.1 Culture and Media

Stock cultures of *Achyla bisexualis* were maintained on the complete nutrient medium PYG (Hintz & Horgen, 1983). For experimental purposes, hyphal mats were grown on

strips of cellophane overlaying PYG medium (2% w/v agar). After cutting hyphae approximately 1 cm behind their apices they were allowed a 1-h recovery period.

4.2.2 Observation Systems

All dynamic experiments were carried out using a Confocal Laser Scanning Microscopy (MRC 1024; Bio-Rad, Mississauga, Ont.) with an Olympus IX70 inverted microscope (NA 1.35, X40, oil iris objective). Relevant effects of osmotica, peptides and inhibitors, growth rate and distances and evaluation of plasmolysis were analyzed on the monitor screen.

4.2.3 Selection and preparation of optimal osmotica

A plasmolyticum is typically chosen to give the easiest and smoothest separation of protoplasts, typically with convex plasmolysis, and ideally without cytomorphological alterations. It should typically be able to pass through cell wall pores yet not be permeable through the plasma membrane. In addition, it should be chemically inert and non–toxic to the cells and should not be metabolised, at least for the duration of the plasmolysis experiment. Sugars (sucrose, glucose) and sugar alcohols (mannitol, sorbitol) are the most frequently used osmotica and seem to meet these criteria, at least for up to a few hours and for most materials (Stadelmann, 1989). Of these sorbitol has been used in a number of studies of plasmolysis in oomycetes (Kaminskyj & Heath, 1995; Kaminskyj *et al.*, 1992b; Money, 1990).

In order to study plasma membrane cell wall attachments we initially tried to use three different concentrations, 0.1, 0.2 and 0.5 M sorbitol made up in SES, to plasmolyse the hyphae of *A. bisexualis* (Figure 4.1; Figure 4.2; and Figure 4.3, respectively). The best concentration of sorbitol was 0.5 M because at this concentration over 50% of the hyphae were plasmolyzed within 30 min (Table 4.1). At 0.1 or 0.2 M of sorbitol concentrations, the cytoplasm contracted back from the tip for a while, it then moved forward to the tip and growth resumed, With these solutions only 1.6% or 6.1% of hyphae showed cytoplasmic retraction and thus plasmolysis within 30 min respectively (Figure 4.1; Figure 4.2). These observations are perhaps not surprising given the observation that most mesophytic cells will plasmolyze in 0.4-0.7 osM (Lee-Stadelmann & Stadelmann, 1989).

4.2.4 The effect of RGDS-containing peptides on plasmolysis

Growing hyphal cultures were treated with a hypertonic solution that comprised, standard extracellular solution (SES; 10 mM KCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM HEPES adjusted to pH 7.0 with 2.5 mM KOH) and 0.5 M sorbitol (Sigma, St. Louis, MO). This was applied to the growth chamber, and drawn through the chamber using filter paper to ensure rapid uniform application of the osmoticum. The patterns of cytoplasmic retraction from the cell wall were recorded as soon as possible after addition of hypertonic solution. The pattern of retraction was recorded as even or uneven. During plasmolysis, indications of uneven retraction were distinct adhesions, remnants of cytoplasm attached to the wall and retraction of cytoplasm in a stepwise manner ie the plasma membrane remained attached to the cell wall on one side of the hypha while on the other side the plasma membrane retracted evenly, until it too reached an attachment point and retraction ceased. If the cytoplasm retracted from the cell wall under equal movement without any plasma membrane sticking on the cell wall, this was classed as even retraction.

For experiments using RGD-containing peptides growing hyphal cultures were initially treated with 0.5 mM, 1.5 mM, 3 mM RGDS, or 3 mM GGR made up in PYG broth for 30 minutes. These were then plasmolyzed with hypertonic solution containing the relevant concentration of RGDS or GGR and the pattern of plasmolysis was observed and recorded as above.

4.2.5 Characterisation of deposited wall material with uneven retraction

In order to identify the material that was deposited when the cytoplasm retracted unevenly, hyphae were plasmolyzed with hypertonic solution (0.5 M sorbitol in SES) for 30 min. The plasmolyzed hyphae were then stained with 0.01% Calcofluor and examined with an Olympus BH2-RFC microscope equipped with an epifluorescence attachment using a mercury lamp. Generally, a Dplan Apo 40 UV, NA 1.0, X40, oil objective was used. Single frame digitized images were recorded with a Nikon D1 Digital SLR.

4.2.6 Characterisation of F-actin organization in plasmolysed hyphae

Hyphae, that had been plasmolysed as described above, were fixed using the methylglyoxal-formaldehyde combination fixative and stained with Alexa-phalloidin as described in Chapter 2.

4.3 Results

The retraction of cytoplasm with plasmolysis was found to be affected by the presence of RGD-containing peptides (Figure 4.4). In the majority of hyphae that were exposed to either RGD or RGDS the cytoplasm retracted from the tip in an even manner without any obvious attachments between the cytoplasm and cell wall (Figure 4.4, Figure 4.5, Figure 4.6). This contrasts with control hyphae that were not exposed to these treatments. In these hyphae retraction was mostly uneven, suggesting the presence of "sticky" spots or adhesion points where attachments are likely to occur between the cell wall and the cytoplasm (Figure 4.4, Figure 4.7, Figure 4.8, Figure 4.9). In some hyphae these attachments remained connected to the main retracting protoplasm via long thin strands of protoplasm that resembled Hechtian strands (Figure 4.7, Figure 4.8). The treatment of GGR was similar to the control (Figure 4.10). The frequency of these is showed in Table 4.2). This suggests the presence of integrin-like proteins within the plasma membrane, which affect adhesion between the PM and cell wall.

4.3.1 Wall material may still be exocytosed despite protoplasmic retraction

In some hyphae retraction of the protoplasm ceased at adhesion sites for several minutes. An example of this is shown in Figure 4.11. Once retraction recommenced in these hyphae and the protoplasm moved away from the attachment sites some material, approximating to the shape of the "protoplasmic tip" remained. This material was found to stain with Calcofluor suggesting that it may be comprised in whole or in part of cell wall material (Figure 4.12).

4.3.2 F-actin localises to wall membrane attachment sites

When plasmolysed hyphae were fixed and stained with Alexa Phalloidin F-actin was found in wall membrane attachment sites (Figure 4.13). At these sites the F-actin was

present in the form of distinct plaques. There was no distinct F-actin cap visible in plasmolysed hyphae, this may indicate the fact that the microfilaments that comprise the cap become disorientated as the protoplasm retracts. Despite the absence of a distinct cap there is a noticeably greater amount of fluorescence in the tip most part of the protoplasm (Figure 4.13B), which may represent the filaments that formed the cap prior to plasmolysis.

4.4 Discussion

The data presented indicate the presence of integrin-like proteins, or at the very least, an RGD-sensitive protein, in hyphae of the oomycete *Achlya bisexualis*. This is indicated by the differences in the numbers of hyphae showing even or uneven protoplasmic retractions upon plasmolysis in the presence of absence of RGD-containing peptides. The data also indicate that these proteins are likely to be present in cell wall-plasma membrane attachment sites that are formed as a consequence of their presence upon protoplasmic retraction. The additional presence of F-actin at these sites suggests that they may link with the F-actin cytoskeleton at these sites.

A number of other studies on tip growing cells of oomycetes, plants and fungi have suggested the presence of cell wall-plasma membrane attachment sites but, as for the growth data that was discussed in Chapter 3, they show differing responses to RGD-containing peptides. Membrane-wall adhesions, which, like those described in this study, contain F-actin have been described in zygotes of the brown alga *Pelvetia* and significantly these were concentrated in the apical most 5 μm of the elongating rhizoid in germinated zygotes appropriate. There were few adhesions along the flanks of the rhizoid or in the thallus region. In common with the data presented in this chapter treatment with RGDS had the effect of reducing the numbers of adhesions. However it is questionable whether these play a role in the tip growth process as there was no correlation between numbers of adhesions and growth rate.

Large cell wall-plasma membrane attachment points and finer attachments similar to Hechtian strands have been reported in the oomycetes *Saprolegnia ferax* and *Achlya ambisexualis* and the ascomycete *Neurospora crassa*. These lacked endoplasmic reticulum but, again like those described in this study, contained F-actin. In contrast to

the present study, however, strand adhesion to the wall was found to be insensitive to RGD-containing peptides (Bachewich & Heath, 1997b). It is possible that the divalent cation concentration may have been insufficient to promote effective binding between the peptide and the integrin-like protein, as has been described earlier in Chapter 3. Clearly there is a future need for a comparative study to be carried out on these respective species in the same laboratory using both the methodology used in the present study and the methodology of (Bachewich & Heath, 1997b).

Cell wall-plasma membrane attachments are not restricted to tip growing cells and have been reported in a number of different plant cells from a number of species. As detailed in the Introduction to the present chapter, these include fern protonemal cells (Kagawa *et al.*, 1992), onion epidermal cells (Oparka *et al.*, 1994; Lang-Pauluzzi, 2000), barley plants (Lee-Stadelmann *et al.*, 1984), *Pteridium* protoplasts (Attree & Sheffield, 1985), protonema of the moss, *Funaria hygrometrica* (Schnepf *et al.*, 1986), oat coleoptile tissue (Drake & Carr, 1978), *Arabidopsis* and *Ginko biloba* callus cells (Canut *et al.*, 1998; (Buer *et al.*, 2000) and in tobacco (*Nicotiana tabacum*) (Pont-Lezica *et al.*, 1993). Their presence in these as well as oomycetes and fungi suggests that integrin-like proteins or homologues of them are present in a variety of evolutionarily distant phyla. Furthermore if they arise due to mechanical attachments that are composed of integrin-like proteins this would also suggest that the integrin-like proteins are common in walled cells.

One interesting observation in the present chapter is that in addition to RGDS, RGD also had an effect on the numbers of cell wall-plasma membrane attachment sites. This contrasts somewhat with the findings presented in chapter 3 where RGDS was found to slow tip growth but RGD was not. The reasons for this discrepancy are at present unclear. One possibility is that an RGD-sensitive component is involved in the formation of cell wall-plasma membrane attachments but is not involved in the process of tip growth. In contrast an RGDS sensitive component appears to be involved in both the formation of cell wall-plasma membrane attachments and in the process of tip growth. Clearly this is an area that warrants further investigation.

Staining of material that was left behind by the "apex of the retracting protoplasm" when it temporarly stopped retracting suggested that this retracting apex is still a site

where exocytosis of wall material is occurring. This is a surprising finding. The F-actin staining of plasmolysed hyphae suggests that the F-actin cap in such sites is disorganised although there does appear to be brighter staining and thus a higher concentration of F-actin relative to the rest of the hypha. It is thus possible to conclude that delivery of vesicles containing wall material to the apex of retracting protoplasm might be perturbed. Clearly some vesicles are still present at the tip and are being exocytosed there as evidenced by the deposition of wall material that was observed. Again this is an area that is ripe for future research.

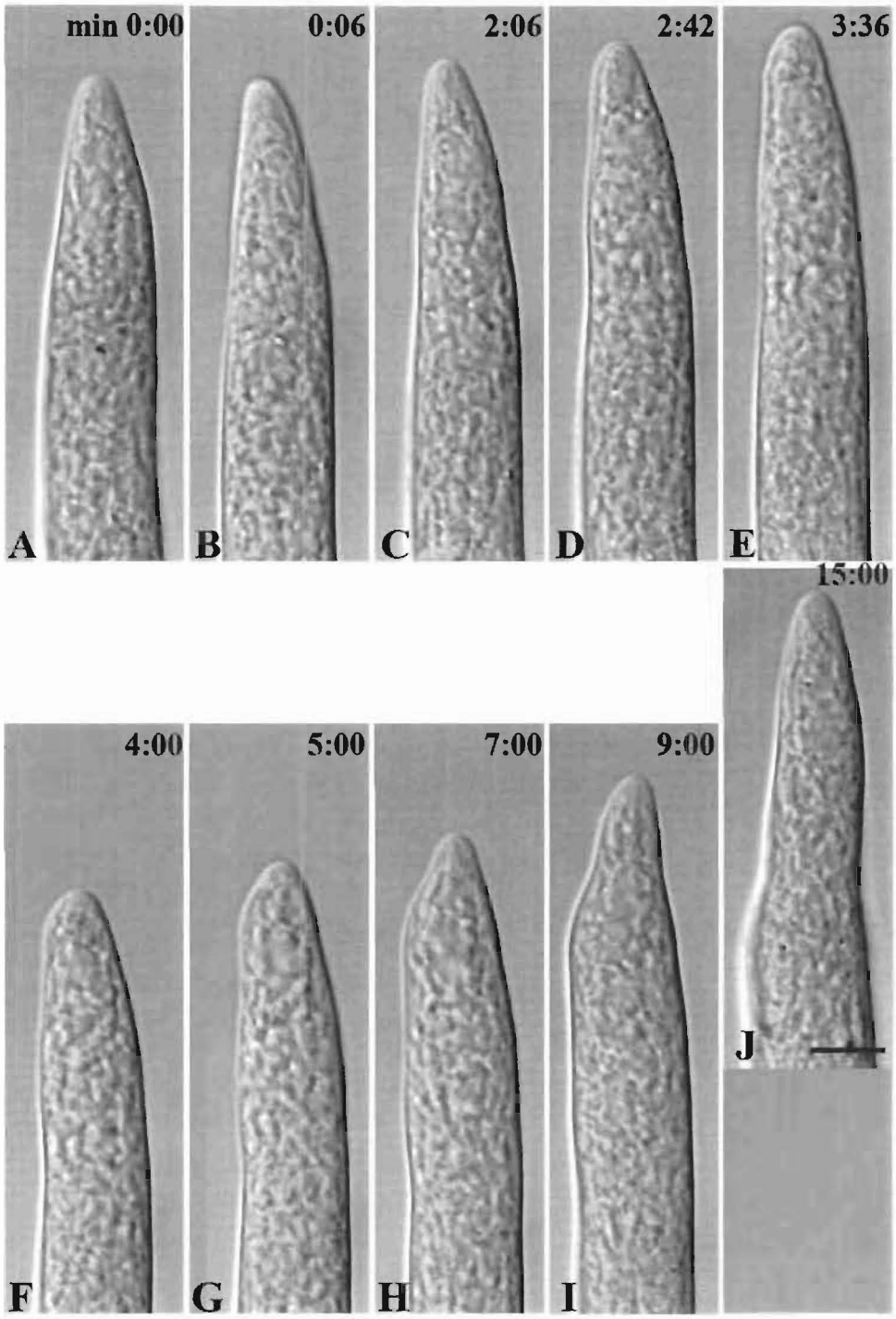


Figure 4.1 Hyphae treated with 0.1 M sorbitol (in SES).

Time labels indicate time after addition of the osmoticum. Bar = 10 μm.

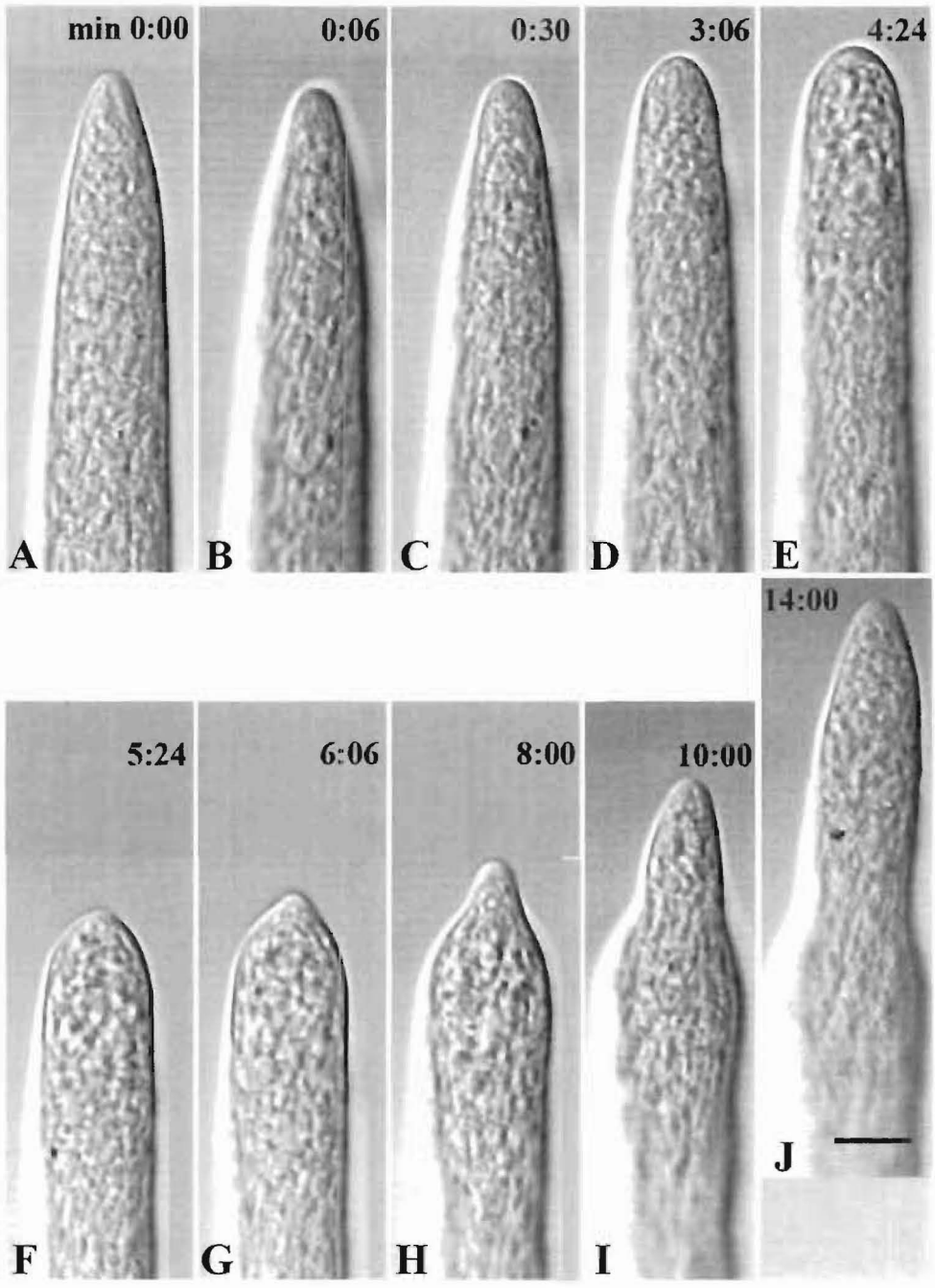


Figure 4.2 Hyphae treated with 0.2 M sorbitol (in SES).

Time labels indicate time after addition of the osmoticum. Bar = 10 μm.

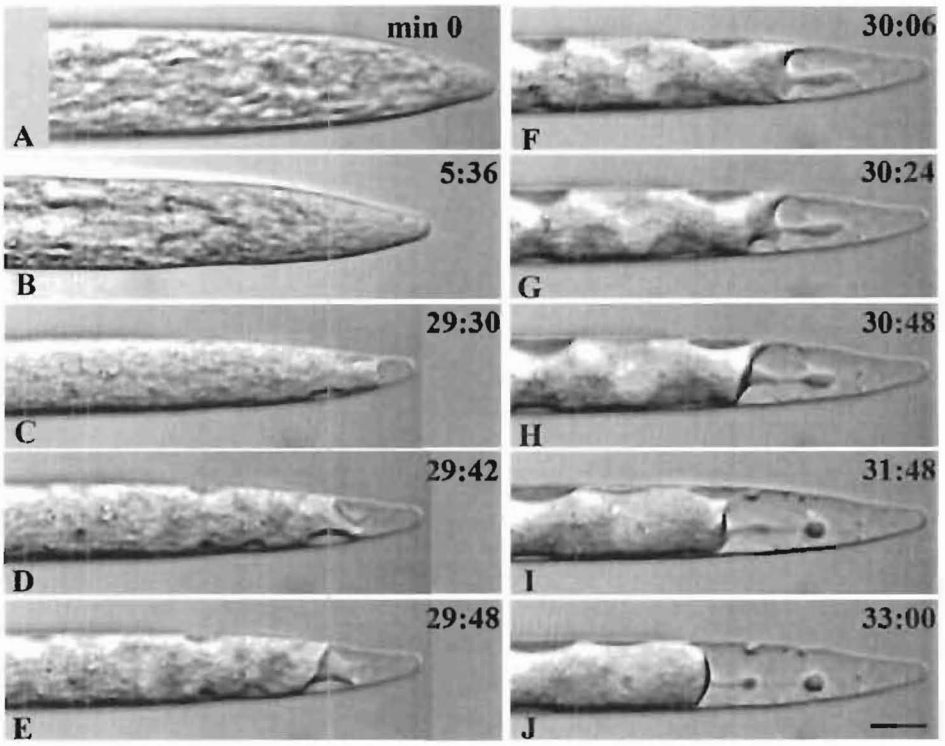


Figure 4.3 Hyphae treated with 0.5 M sorbitol (in SES).

Time labels indicate time after addition of the osmoticum. Bar =10 μm.

Table 4.1 The effect of different sorbitol concentrations on plasmolysis

Concentration	Plasmolysis	Normal growth	Stop then growth	Stop or round tip	No. of hyphae
		%			
0.1 M	1.6	94.3	0	4.1	120
0.2 M	6.1	6.1	73.7	14.1	99
0.5 M	81.7	0	0	18.3	104

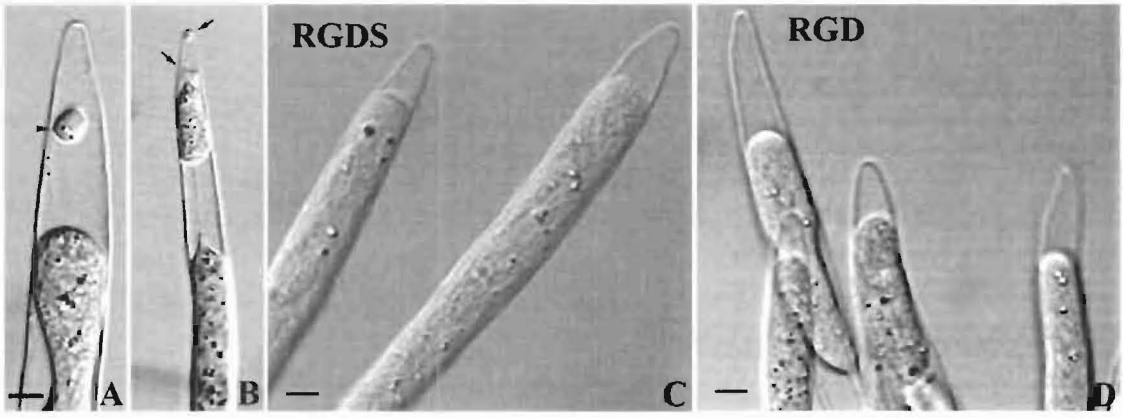


Figure 4.4 The effect of RGD-containing peptides on patterns of protoplasmic retraction

In the absence of RGD-containing peptides (A, B) there were some adhesion attachments (arrow in B) and blebs (arrowhead in A) formed at the subapical regions and some remnants at the extreme tip. C-D Hyphae that were plasmolysed in the presence of RGDS or RGD presented even convex forms without any obvious cytoplasmic remnants at the tip. Bar = 10 μm.

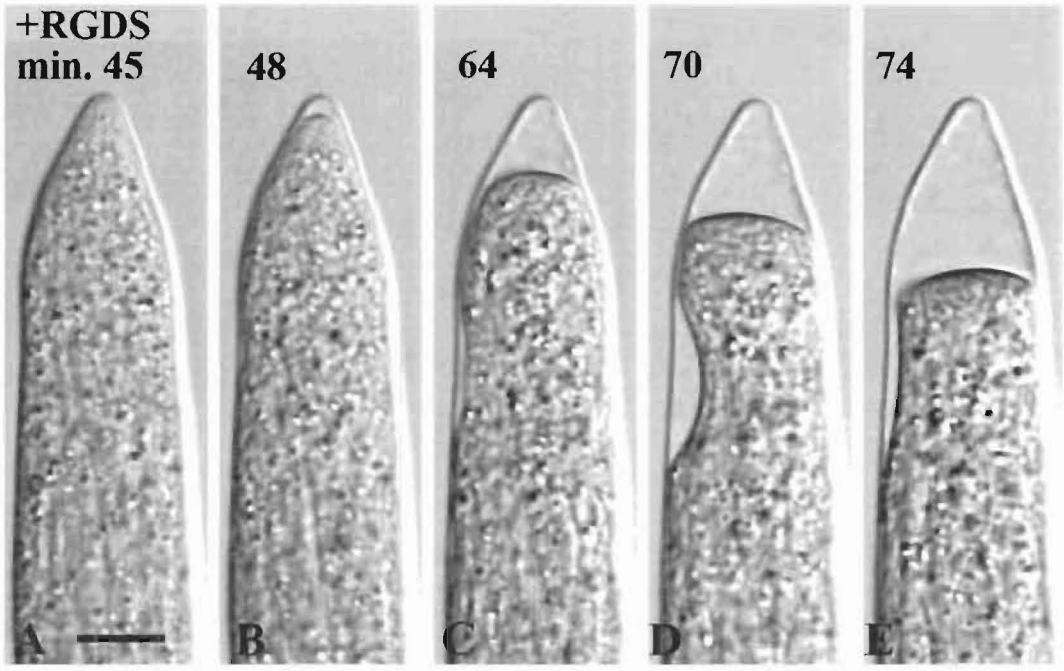


Figure 4.5 RGDS induced even retraction of the protoplasm during plasmolysis.

Bar = 10 μm.

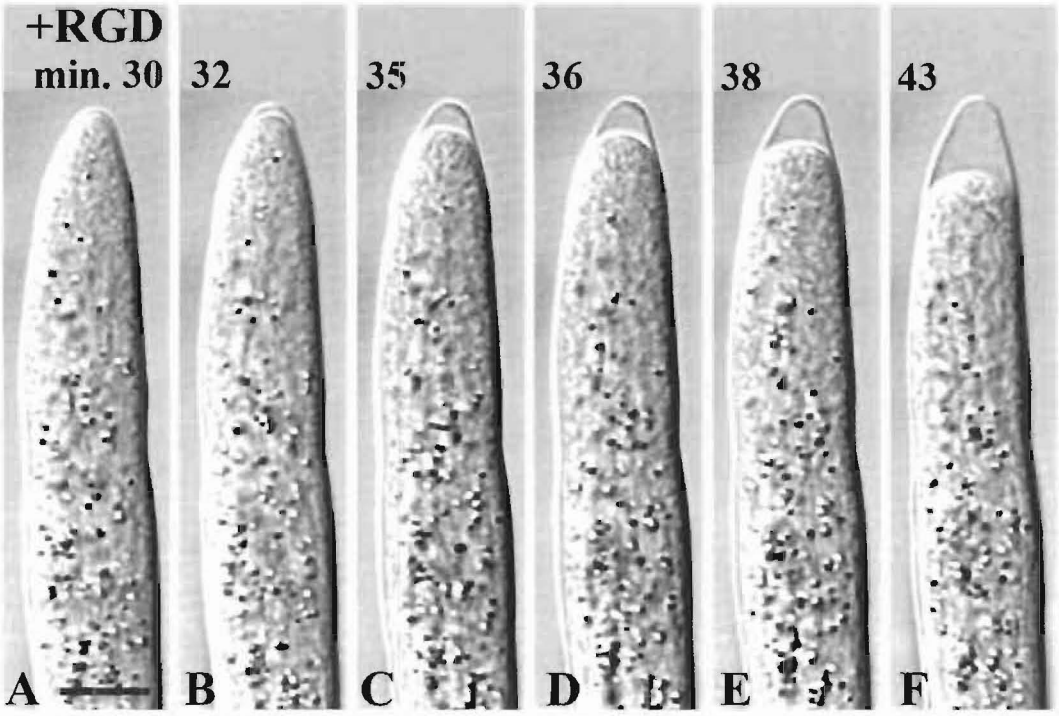


Figure 4.6 RGD induced even retraction of the protoplasm during plasmolysis.

Bar = 10 μm.

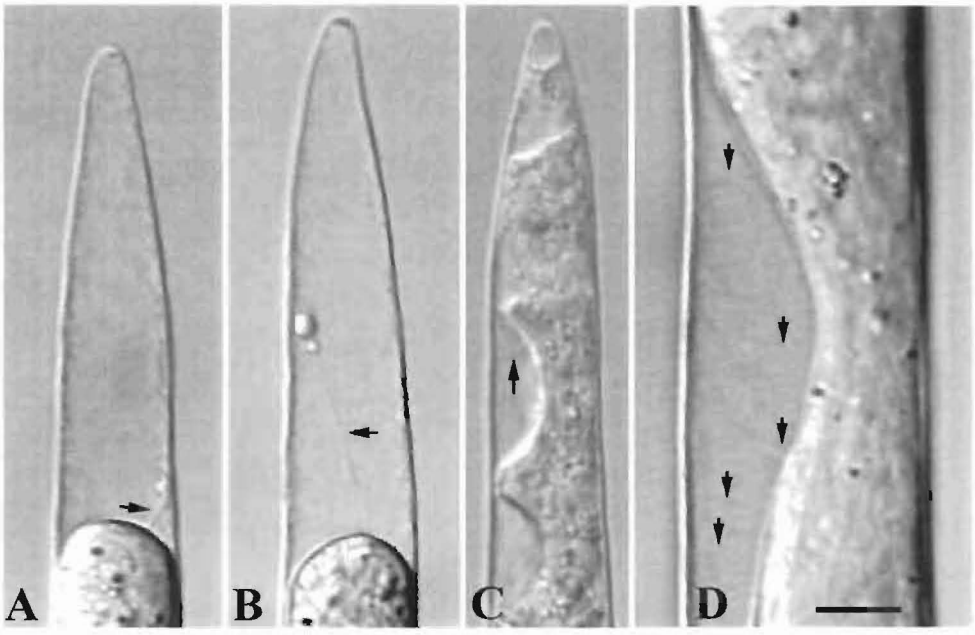


Figure 4.7 Hechtian-like strands formed at the tip or subapical regions of plasmolysed hyphae (indicated by the arrows).

Bar = 10 μm.

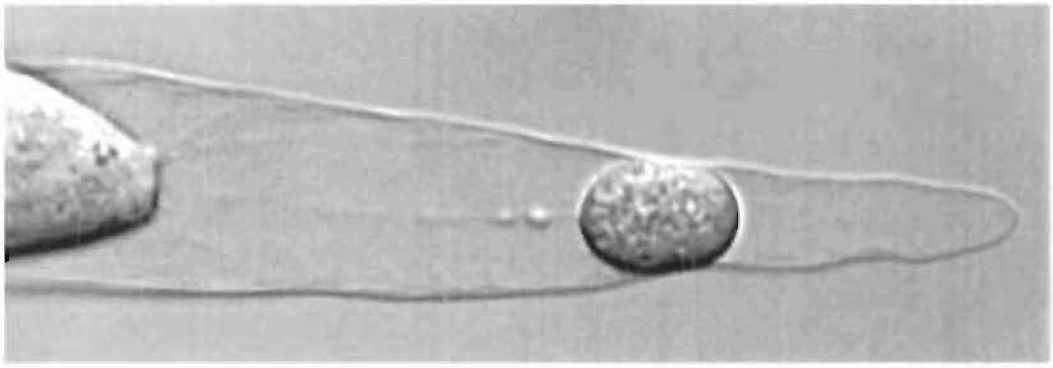


Figure 4.8 Formation of Hechtian-like strands in the subapical region of a plasmolysed hypha

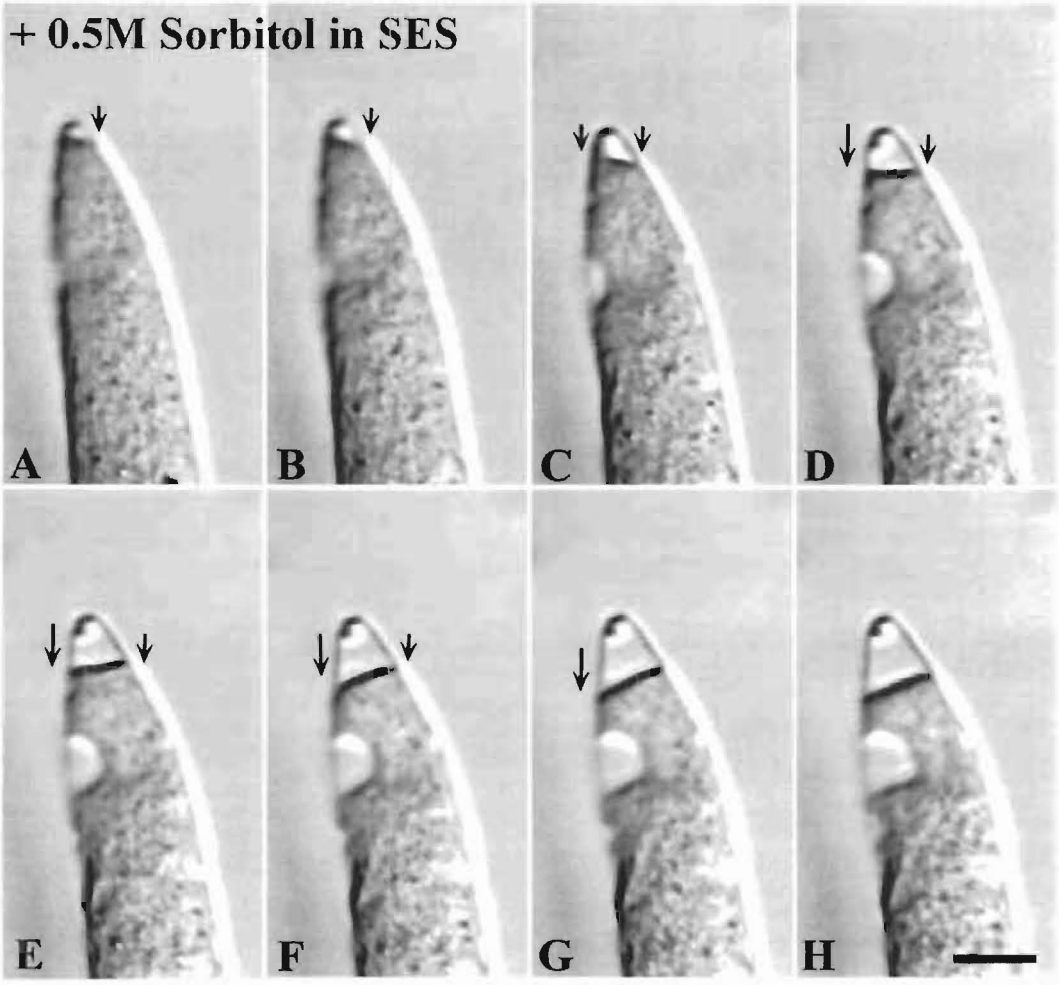


Figure 4.9 Uneven retraction of protoplasmic retraction

The hypha was treated with 0.5 M sorbitol (in SES), it caused the cytoplasm to retract unevenly along one side of the hypha and then the other. The longer arrow indicates faster retraction. Bar = 10 μm.

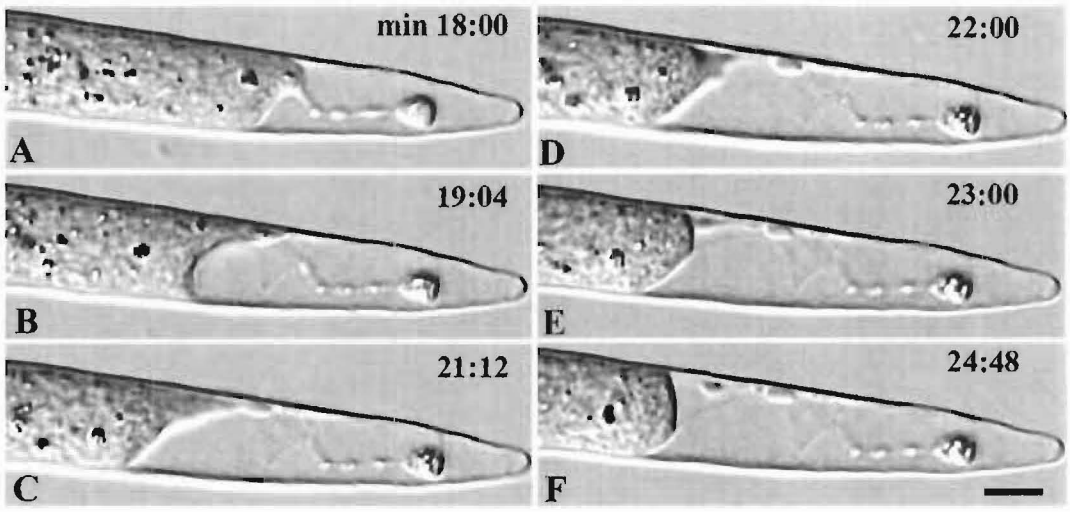


Figure 4.10 GGR treated hyphae showed uneven protoplasmic retraction and blebs.

Bar = 10 μm.

Table 4.2 The effect of RGD-containing peptides on the pattern of protoplasmic retraction

Pattern / Treatment	Uneven retraction %	Even retraction %
Sorbitol (n=365)	86	14
Sorbitol with GGR (n=243)	66	34
Sorbitol with RGD (n=243)	25	75
Sorbitol with RGDS (n=358)	5	95

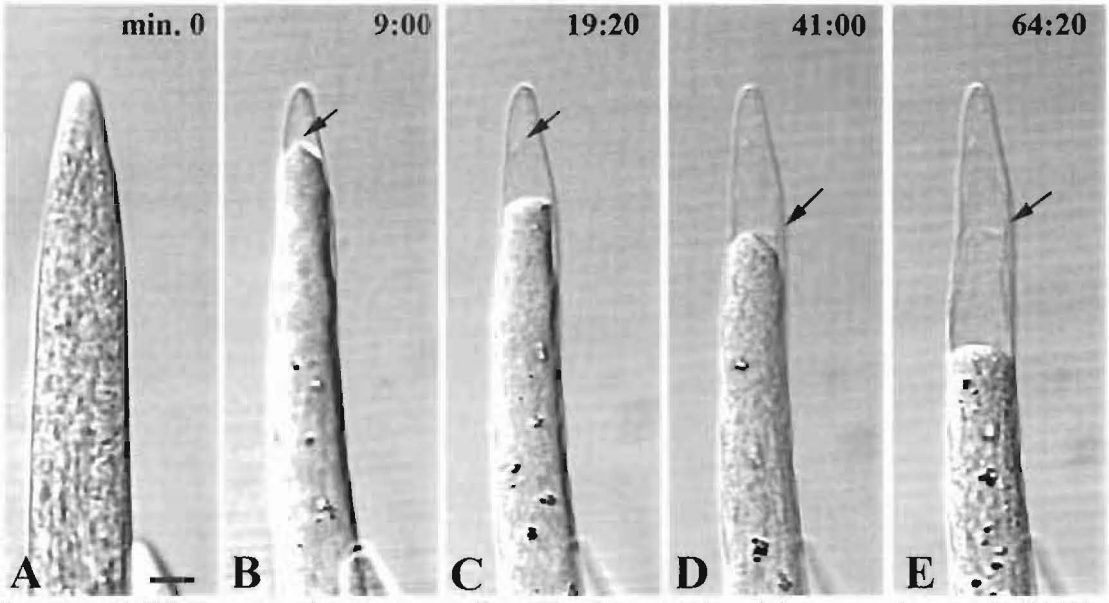


Figure 4.11 When retraction temporarily ceased at certain sticky spots (arrow in B, D) there was deposition of material at the leading edge of the protoplasm in the shape of the protoplasm (arrows in C, E).

Bar =10 μm.

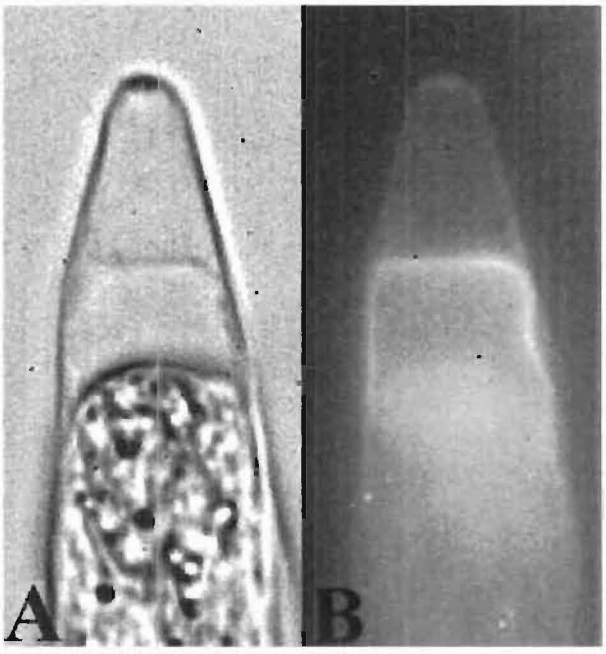


Figure 4.12 Material that was deposited at the tip of retracting protoplasm stained with Calcofluor, which is suggestive that it is wall material.

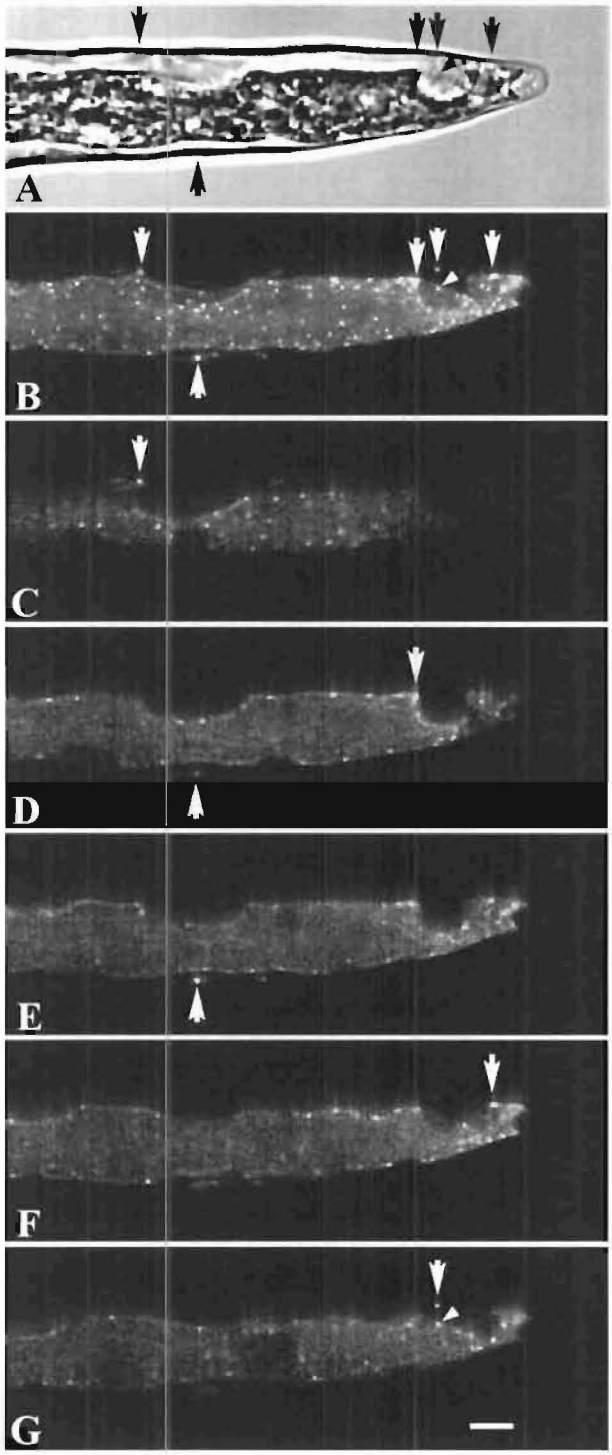


Figure 4.13 F-actin is present in wall membrane attachment sites, A DIC images of plasmolysed hypha, B Z series, C-G series sections (arrows indicate plaques at attachment points).

Bar = 10 μm.

Chapter 5

The effect of RGDS on the mycelial morphology of *Achlya bisexualis*, as determined by fractal analysis

5.1 Introduction

In any study of oomycete and fungal growth it should be remembered that these organisms exist for the most part, not as single hyphae, but as a collective. The morphology of a collective of hyphae or mycelium is determined by mechanisms, which regulate the polarity and the direction of growth of the hyphae and the frequency with which they branch. If integrin-like proteins play a role in regulating the process of tip growth, as earlier chapters of this thesis suggest, then it is likely that they will influence the morphology of a mycelium. Such regulatory mechanisms are thus likely to make a significant contribution to the efficiency with which oomycetes and fungi colonise solid surfaces. Observation of a colony developing on a solid rich medium shows that hyphae grow radially outward from the inoculum with leading hyphae at the colony margin growing approximately parallel to one another and at approximately the same distance apart.

In nature the situation is more complex with two types of growth strategies likely to be utilised by hyphal organisms. A phalanx growth strategy is characterised by mycelia that can be described as tightly aggregated rosettes of hyphae. These are typically slow growing and develop in rich media substrates. Such broad-fronted, relatively slow growing mycelia are thought to utilise diverse and locally abundant nutrient resources.

Phalanx type growth approximates to the pattern of growth of a mycelium grown on a petri dish that contains a nutrient enriched media in the lab, as described in the preceeding paragraph. In contrast, the guerilla growth strategy is characterised by the development of loosely aggregated, fast-growing branches within mycelia. This type of growth is likely to occur when the organism is growing in an environment that is nutrient poor and has been hypothesised to be a foraging, or indeed an escape mechanism (Carlile, 1995). Mycelia of different species exhibit characteristic growth patterns, and such mycelial systems can alter their morphology under different growth conditions or in response to localised or dispersed nutrients. Thus, mycelial morphology is influenced by both the species in question and by the environmental conditions. Neither strategy should be seen as mutually exclusive and it is likely that they represent the extremes of what is a continuum of growth types.

In addition to nutrient availability, the presence of toxic compounds such as heavy metals can also affect morphology. Cadmium for example, was shown to affect radial growth rate and polarized growth of *Schizophyllum commune* (Lilly *et al.*, 1992) and copper affects mycelial length, number of branches and biomass distribution of *Trichoderma viride* and *Rhizopus arrhizus* (Ramsay *et al.*, 1998). The species of interest in this thesis *A. bisexualis* typically produces more-or-less straight, subapically branched, hyphae when growing in liquid or agar-solidified media, with abundant aerial mycelium on the latter (Harold, 1994). Mycelial morphology has been shown to be significantly affected by heavy metal challenge (Lundy *et al.*, 2001).

As mycelia are irregular in shape this raises a problem with regard to their study. Irregularity rules out standard Euclidean descriptives that would assign whole number dimensions of 1 (straight line), 2 (flat surfaces) and 3 (volumes) (Schmid & Harper, 1985). Researchers have thus tended to quantify such things as radial extension, biomass, hyphal surface area cover and density. A more recent approach has been that of fractal geometry. This is a means of determining the degree to which a pattern or structure is self-similar, i.e., similar at different scales of measurement. The fractal dimension obtained from fractal analysis quantifies self-similarity such as border fractal dimension (D_{border}) and also provides a measure of the space-filling capacity of the structure such as mass fractal dimension (D_{mass}). This method has been used in studying

fungal and oomycete growth in response to different environments (Donnelly *et al.*, 1999; Lundy *et al.*, 2001; Prosser, 1994).

As described in earlier chapters RGDS can result in the slowing or cessation of growth in a concentration-dependent manner and can affect hyphal morphology. It is thus reasonable to predict that it may likewise affect mycelial morphology. In this chapter the effects of RGDS and other growth inhibitors such as TPA, on the morphology of mycelia as determined by fractal geometry and by measurement of mycelial surface area and radial extension is investigated.

5.2 Materials and methods

5.2.1 Cultures and media

Cultures of *A. bisexualis* were maintained as described in Chapter 2.

Due to the prohibitive cost of using RGDS containing peptides in Petri dish cultures a smaller growth chamber was made that comprised a microscope slide with a circular hole cut from the middle. The base of the chamber was made by sticking a cover slip to the bottom of the slide using Vaseline. These chambers were filled with the relevant growth media and were inoculated with a mycelial plug with a radius of 2 mm that was taken from stock plates approximately 1 cm behind the growing edge of the colony.

Hyphae were grown on dialysis tubing that overlaid the agar. This was necessary to prevent hyphae penetrating the medium and effectively growing as a three dimensional entity. This is essential as the technique of fractal geometry is limited to a two dimensional image. Thus by growing on dialysis tubing overlaying the agar media the mycelia are assuming as close an approximate as possible to a two-dimensional structure. Prior to use the dialysis tubing were cut into squares and boiled 3 times in distilled H_2O on a stirring hot plate with stirring bar in order to remove any bactericides and manufacturing residues. Without this treatment growth is severely restricted. The dialysis tubing squares were placed in a tissue culture pot and autoclaved 20 minutes at 121°C and stored in a fridge prior to use.

5.2.2 Image capture and pre-process

After inoculation and growth at 20°C for 12, 24, 36, 48 hour-periods mycelia were imaged, binarised, and images adjusted according to the methodology of (Lundy *et al.*, 2001). Basically imaging was accomplished using a Microtek Scanmaker V6USL (Microtek Lab Incorporated) set to a fixed image size and fixed resolution. The digitised images were then binarised using Adobe Photoshop 5.0 (Adobe Systems Incorporated) software prior to fractal analysis. The first step in this process was to improve the quality of the image by adjusting brightness and contrast settings to increase the contrast between the mycelium and the medium (background). Conversion into binarised images was accomplished by manually adjusting the threshold value. The pixels at or above the threshold intensity were set to white (a greyscale value of 255), this representing the mycelium, and pixels below the threshold intensity were set to black (a greyscale value of 0), representing to the background (medium) (Figure 5.1). Any pixels that were obviously not part of the mycelia (for example the edge of the Petri dish, bubbles in the agar) were manually removed and set to black (ie a greyscale value of 0). Finally, for the purposes of the *ImageJ* software (details which are given below), the image (white pixels) was converted to black and the background was converted to white for fractal analysis. Any images that could not reproduce the original digitised images were discarded and not used (for example, in some instances it was not possible to remove artefacts, such as when the mycelial edge was close to the edge of the growth chamber or Petri dish).

5.2.3 Fractal analyses

Fractal analyses and calculation of mycelial area were carried out using *ImageJ* software. This is available in the public domain via the NIH website (from website address fttp://rsbweb.nih.gov/pub/image-J/). Mycelial areas were calculated by counting the number of black pixels in each image (this is done automatically in *ImageJ*) and multiplying this by the area that each pixel represented. The area of the inoculation plug was subtracted from the obtained value. Fractal dimensions were determined using the box counting method in *ImageJ*. To accomplish this methodology *ImageJ* overlays the binarised image of the mycelia with grids of varying dimensions (for example square boxes with a width of 3, 6, 12, 24, 36, 48 and 60 pixels). For each size the number of

boxes that intersect the image is automatically counted. Fractal dimensions are calculated using the fact that fractal images obey a power law relationship over a particular range of scales. Thus $N(s) = cs^{-D_B}$ where N(s) equals the total number of boxes of side length s that intersect the image and c equals a constant. D_B represents the border fractal dimension and can be obtained as the gradient of a plot of log N(s) against log (s). Values close to 1 represent a relatively smooth border (approximating to a phalanx growth strategy); values approaching 2 represent a more serrated border (approximating to an increasingly guerilla type growth strategy). The border fractal dimension, which requires only pixels that represent the margin or border of the mycelium, can be easily obtained using an outline function in *ImageJ* that elimates all black pixels that do not represent the margin (Lundy *et al.*, 2001).

Obtained data was analysed statistically using the software package Statistix 7 (Analytical Software). All treatments were compared using one-way ANOVA and Turkey tests.

5.2.4 Observation Systems

The hyphal morphology was recorded using Confocal Laser Scanning Microscopy (MRC 1024; Bio-Rad, Mississauga, Ont.) via an Olympus IX70 (inverted) microscope with a NA 1 X10 or X20 objective. Single frame digitized images of the hyphal morphology was recorded using a Nikon D1 Digital SLR camera connected to an Olympus BH2-RFC microscope equipped with a Dplan Apo 10, NA 1.0, X4 or 10, objective. Photomicrographs of the mycelia not used for fractal analysis were taken on a Wild stereomicroscope (Wild photomakroskop M400, Heer Brugg, Switzerland).

5.3 Results

5.3.1 Comparison of the slide chamber and petri dish methods

In studies of the fractal nature of mycelial growth mycelia are typically inoculated and grown in relatively large growth chambers or in Petri dishes (see for example Donnelly et al., 1999; Lundy et al., 2001). In this latter study, looking at the effect of heavy metals on the morphology of *A. bisexualis*, a standard 9 cm diameter petri dish was used.

The prohibitive cost of RGD containing peptides meant that an alternate smaller growing chamber was required. Initial experiments were therefore carried out that investigated the feasability of using slide growth chambers that were made from a standard microscope slide with a circular hole drilled through the middle. The base of the chamber was made with a cover slip that was adhered to the bottom of the slide using Vaseline. In Figure 5.1and Figure 5.2 comparative images are shown that suggest that the type of growth chamber used can significantly affect the observed morphology. Respective measures of surface fractal, area and radial extension are shown graphically in Figure 5.3and Figure 5.4. The data indicate that, mycelia grown on the slide chamber have more irregular edges, as evidenced by the significantly higher surface fractal values at 36 and 48 hours ($P<0.001$ and $P<0.0001$ respectively, $n=8$) in Figure 5.3 relative to Figure 5.4. In addition at 36 and 48 hours the mycelial area was significantly lower for those mycelia grown in the slide chamber compared to those grown on the Petri dish ($P<0.05$ and $P<0.005$ respectively, $n=8$). The radial extension of the mycelium was also significantly lower on the slide chamber after 48 hours ($P<0.05$, $n=8$). The border fractal of mycelia grown in the slide chamber showed a significant increase after 36 and 48 hours compared to 12 and 24 hours ($P<0.0001$; $n=8$) (Figure 5.3). This increase was not evident in Petri dish grown mycelia.

5.3.2 The effect of RGDS on mycelial morphology

In comparison to the control slide, RGDS was found to significantly decrease mycelial area (at 48-hr, $P<0.05$, $n=8$) and radial extension (at 48-hr, $P<0.05$, $n=8$). This is not surprising given its effect on hyphal growth rate as described in earlier chapters. It had no significant effect on border fractal ($P>0.05$).

As has been detailed in earlier chapters the inclusion of the additional controls of acetate and non-RGD containing peptides is necessary when studying the effects of RGD-containing peptides. Neither of these were found to differ significantly from the control ($P>0.05$, $n=8$), nor was an additional control of SES solution ($P>0.05$, $n=8$).

In order to investigate whether the effects of RGDS are due to a slowing of growth and/or pertubation of the relationship between tip growth and branching an additional treatment of TPA was tested, at concentrations that slow growth to a similar rate to that

observed with 1.5 mM RGDS. The major finding with this treatment was that there was in comparison to the control a decrease in mycelial area ($P<0001$, $n=8$) and radial extension ($P<0.0001$, $n=8$) as might be expected for agonists that slow tip growth. Thus, these effects are similar to that observed for RGDS. In contrast to RGDS however there was, after 48 hours, a statistically significant decrease in the border fractal ($P<0.0001$, $n=8$). The border fractal of mycelia that had been grown in a Petri dish did not differ significanlty from the control, this is likely a result of the consistent border fractal of the control treatments (Figure 5.4). The various responses are summarised in Table 5.1. In addition the Table summarises observations that were made on hyphal diameter and the amount of branching.

5.4 Discussion

This study is to the best of my knowledge, the first to investigate the effect of RGDS on the mycelial morphology of either an oomycete or a fungus. As detailed above oomycetes and filamentous fungi grow as radially extending tubular hyphae that branch and interweave to form as a mycelium. The morphology of a mycelium is therefore dependent upon a number of factors that include the rate and the direction of tip growth, the branching frequency and the angle at which the branch forms. The data indicates that RGDS can significantly decrease mycelial area and radial extension but has no apparent effect on the border fractal. Thus it appears that RGDS will affect the rate of growth but does not significantly affect the relationship between tip growth and branching. This is of interest in that many agents that slow tip growth will also increase the rate of branching (see for example Table 1 in Jackson *et al.*, (2001)). This has been used to suggest an intricate link between the processes of tip growth and branching, which seems logical. It might be predicted therefore that RGDS in slowing growth would decrease the border fractal as an increase in branching would give rise to a smoother edged mycelium yet this is clearly not the case. Critically TPA, which also slows growth, decreases (for mycelia grown in slide chambers) border fractal. Therefore RGDS is acting in a manner that differs from other agents that simply slow growth. This may suggest that integrin-like proteins may also be important with regard to regulating the relationship between tip growth and branching in addition to playing a role in just the tip growth process itself.

Jackson *et al.*, (2001) suggested a model for the formation of a branch that is based on a sequential progression. First, a hypha perceives and transducts some form of directional cue from either internal or external factors. An internal factor is suggested with the concept of the hyphal growth unit, which proposes that a certain volume of cytoplasm is required to support a growth tip. As this volume increases there is a concurrent increase in the number of sub-apical vesicles, which are deposited at the side of a hypha thus giving rise to a branch (Trinci *et al.*, 1994). Hyphae also respond to external factors such as nutrients, substrate texture, moisture, gravity, electrical field and light all of which could act as branch cues (Alexopoulos *et al.*, 1996). These have been shown to affect the development of polarity in fucoid zygotes, a process that is analogous to the formation of a branch (Kropf *et al.*, 1999). Exteral factors such as applications of amino acids or UV irradiation can also influence the hyphal growth unit.

How then might the branching cue be transduced such that the process of branching is set in motion? Jackson *et al.*, (2001) suggest that this may require positional information that could be provided by a molecule or processes that act as a morphogen. Candidate morphogens include Ca^{2+} gradients (Jackson & Heath., 1989), transcellular ion currents or proteins that are analogous to those involved in bud formation in yeast. With regard to tip growth calcium ions serve as a signal that localizes the tip and promotes localised exocytosis (and thus the deposition of new plasma membrane and cell wall). The additional involvement of Ca^{2+} in branching has been suggested in a number of studies (Dicker & Turian, 1990; Robson *et al.*, 1991a; Robson *et al.*, 1991b; Schmid & Harold, 1988) (Harold & Harold, 1986; Reissig & Kinney, 1983). For example in *N. crassa* treatment with the ionophore A12387 increased intracellular [Ca^{2+}] and induced branching (Reissig & Kinney, 1983). There is some controversy however as Ca^{2+} ions may act as a morphogen in the UV irradiation induced branching response for hyphae of *Saprolegnia ferax* (Hyde & Heath, 1997), but it is not the case for *Achlya bisexualis* when phenylalanine is used as the branching cue (Jackson *et al.*, 2001). In eukaryotic cells, calcium acts as a second messenger. Transduction of an external stimulus (e.g., from a hormone) elevates intracellular calcium levels either by stimulating the breakdown of phosphatidylinositol bisphosphate, the generation of IP_3 and the triggering of the release of calcium from intracellular stores, and/or by opening of voltage-dependent calcium channels in the membrane. The effects of increases in the

level of intracellular calcium are mediated via calcium-binding proteins such as calmodulin (CaM). Inhibitors of CaM have been shown to affect branching in the fungus *Fusarium graminearum* (Robson *et al.*, 1991a).

Growing hyphae of *Achlya bisexualis* drive a transcellular current from tip to trunk, positive charge enters the apical zone and leaves distally (Kropf *et al.*, 1983). The flow of charge through the hypha is due to partial segregation of proton pumps from proton-coupled transport systems. There have been suggestions that this may act as a morphogen as the zone of inward current has been found to precede the emergence of a branch and predicted its approximate location (Kropf *et al.*, 1983; Kropf *et al.*, 1984). This is an area of some controversy however as the current is not always present (Cho *et al.*, 1991; Harold, 1994).

Finally the suggestion of proteins such as the rho GTPase Cdc42p acting as a morphogen is supported by its accumulation at future bud sites in yeast, and its subsequent control of the actin cytoskeleton. Cdc42 is highly conserved among fungi, yet there has been debate as to whether its role in the development of polarity in filamentous species such as *Aspergillus nidulans* is as important as that in yeast (Harris & Momany, 2004).

In the model of Jackson *et al.*, (2001) once postional information, in the form of a morphogen, is established this leads to the recruitment of F-actin and associated accessory proteins that then enable the formation of a bump that subsequently develops into a branch. In establishing such polarised growth the F-actin is likely to function in vesicle delivery and controlling vesicle fusion at the plasma membrane (Gupta & Heath, 1997; Heath, 1995; Jackson & Heath, 1990a; Kaminskyj & Heath, 1996). This is the first visible sign of branching.

RGDS slows growth, yet has no effect on border fractal dimension and therefore branching while most other agents (including TPA), slow growth and increase branching. This suggests that integrin-like proteins may play a role in coupling the processes of tip growth and branching or that they may inhibit branching and thus negate the normally increased rate that is observed with the slowing of growth. The former could occur if perturbation of the arrangement of the F-actin cytoskeleton

affected the hypha's perception of, and ability to control, the hyphal growth unit. With regard to the latter possibility, this suggests a more direct role for integrin-like proteins in the process of branching. One such role could be the facilitation of the arrangement of F-actin in the correct pattern that would enable bump formation. This then raises the possibility that the integrin-like proteins themselves may act as morphogens. Certainly the observation, in both *Saprolegnia ferax* and *Neurospora crassa,* that these proteins localize to the hyphal tip is consistent with such a role (Degousee *et al.*, 2000; Kaminskyj & Heath, 1995). In inhibiting the "increased branching" process RGDS will lead to an increase in border fractal dimension, which will, with the concurrent slowing of hyphal growth rate, decrease mycelial area.

It is also possible that RGDS will affect other proposed morphogens such as Ca^{2+} and transcellular ion currents. The distribution of ion channels and possibly other membrane transport proteins have been shown to be affected by agents that perturb F-actin distribution (Levina *et al.*, 1994). This suggests that F-actin may play a role in positioning these proteins in polar cells and this may arise due to the positioning of F-actin itself through its interations with integrin-like proteins. RGDS may thus affect ion gradients by perturbing the normal interactions of the integrin-like proteins.

Clearly the above is an area where more work is warranted. It should be noted, however, that studies on branching are difficult, this is especially the case for the very early stages of the process, as it is only possible to, at best estimate, where a branch will form. The development of a localised branch induction technique such as that described by (Jackson *et al.*, 2001), while fraught with difficulties may in the future lead to a clearer understanding of the process.

Finally this chapter presents a cautionary note for studies of mycelial morphology in that the type of growth chamber used can significantly influence the observed morphology. Clearly in carrying out future studies it will be prudent to use one type of growth chamber.

Table 5.1 Summary of the different types of response to the various treatments

Treatment	Area and radial extension	Border fractal	Branching	Diameter of hyphae
Control, Kac, GGR (on slide chamber)	normal	normal	normal	normal
SES (on slide chamber)	no effect	no effect	no effect	no effect
RGDS (on slide chamber)	decreased	no effect	no effect	no effect
TPA (on slide chamber)	decreased	no effect	increased	increased
Control , Kac, SES (on Petri dish)	normal	normal	normal	normal
TPA (on Petri dish)	decreased	decreased	increased	increased

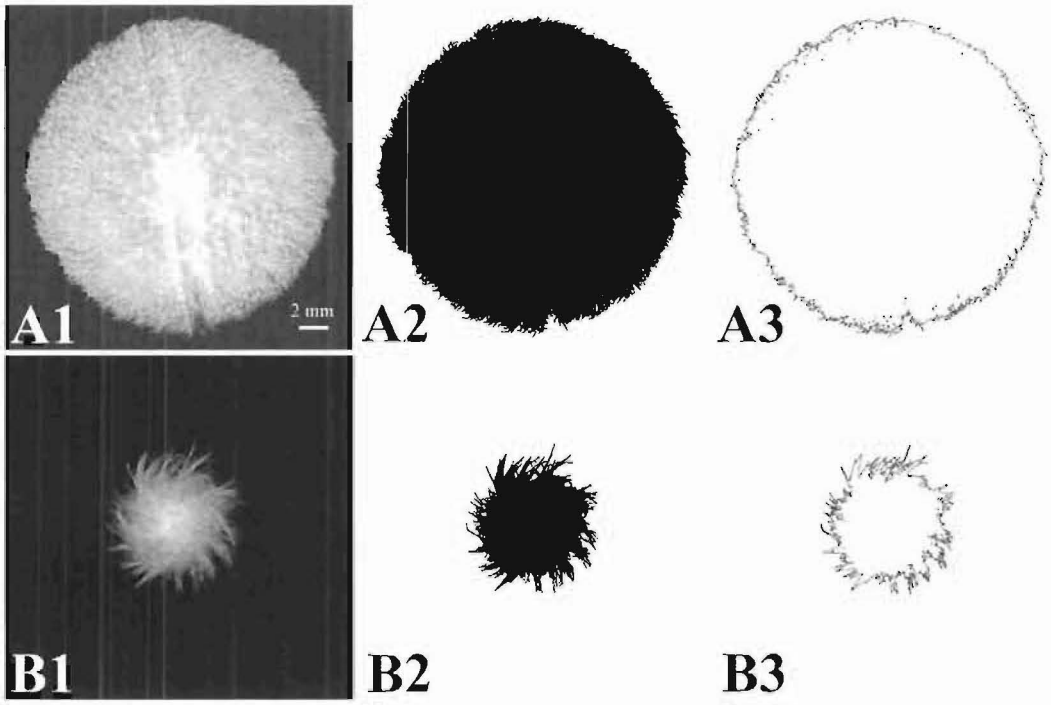
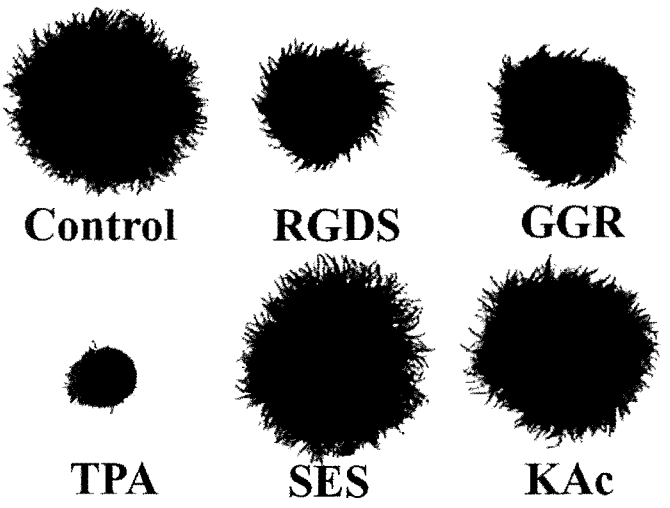
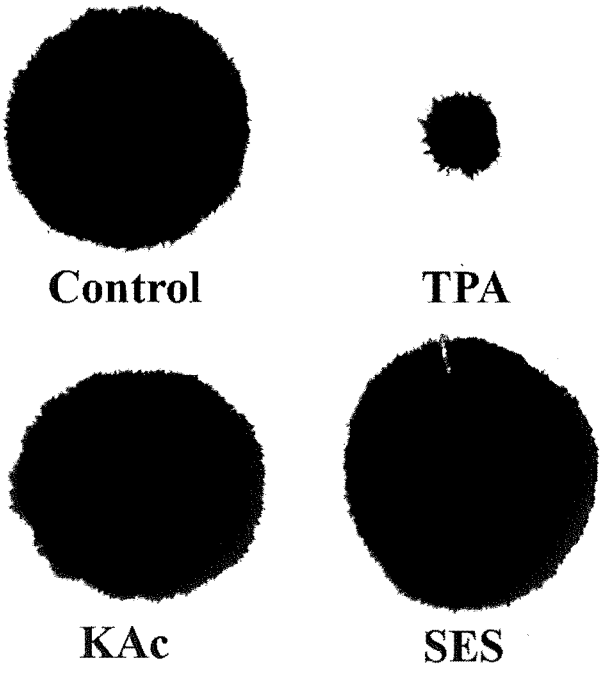


Figure 5.1 Digitalised (A1, B1) and binarised (A2, B2) and outline (A3, B3) images of a mycelium grown in a Petri dish (A) and in a slide chamber (B).

Hyphae extending out from the inoculation plug formed a circular mycelium with relatively smooth edges in the petri dish. Behind the mycelial edge, hyphae completely covered the media. In contrast, hyphae grown in the slide chamber, formed a circular mycelium with relatively irregular edges.



A. Grown on the slide chamber overlaid with dialysis membrane



B. Grown on the Petri dish overlaid with dialysis membrane

Figure 5.2 Binarised images of mycelia grown in a slide chamber or Petri dish in which the PYG media was supplemented with the chemical(s) indicated. Clearly the growth chamber used can influence the mycelial morphology.

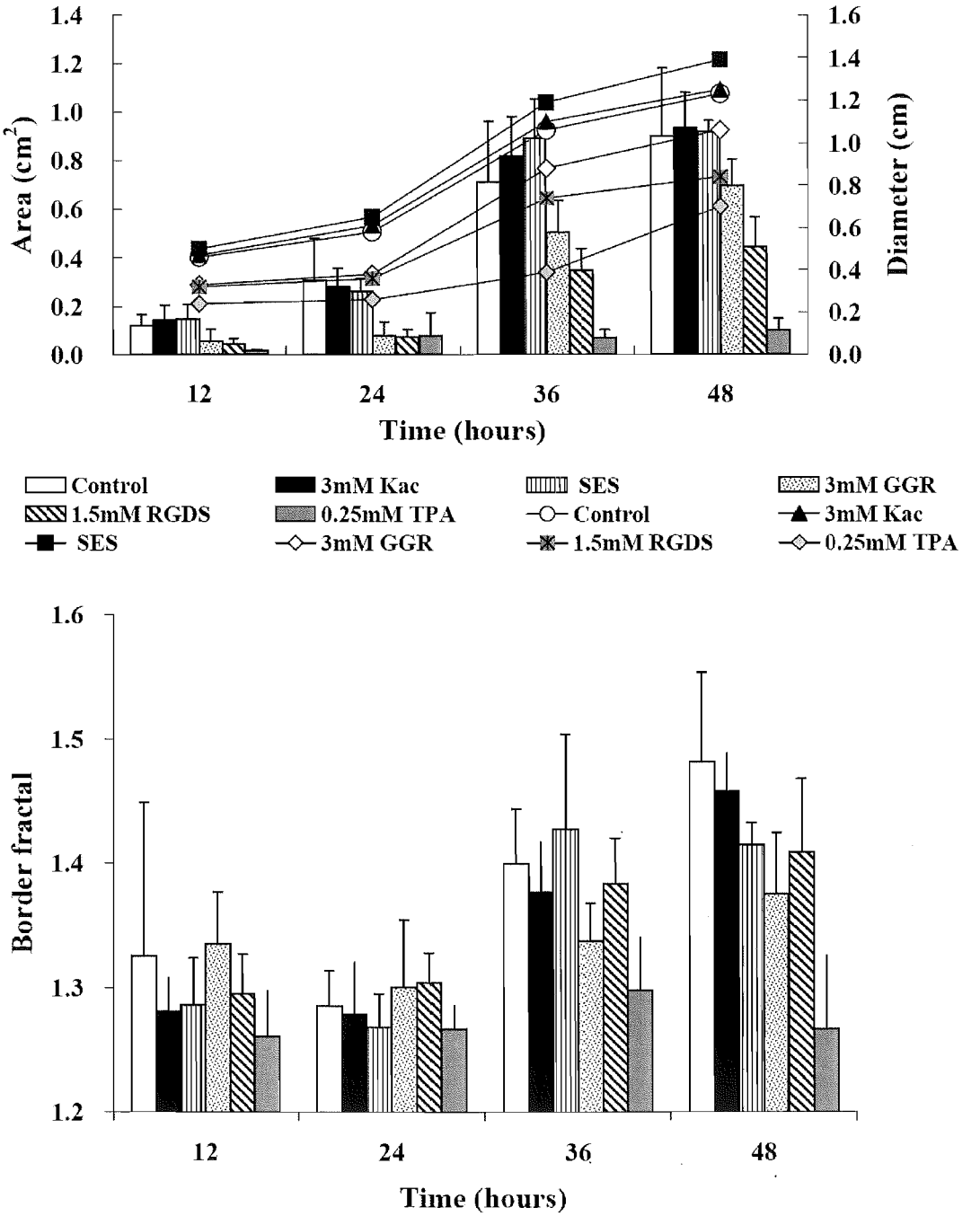


Figure 5.3 The effect of RGDS and TPA, on mycelial area (bar), radial extension (line) and border fractal dimension (bar) of slide chamber grown cultures.

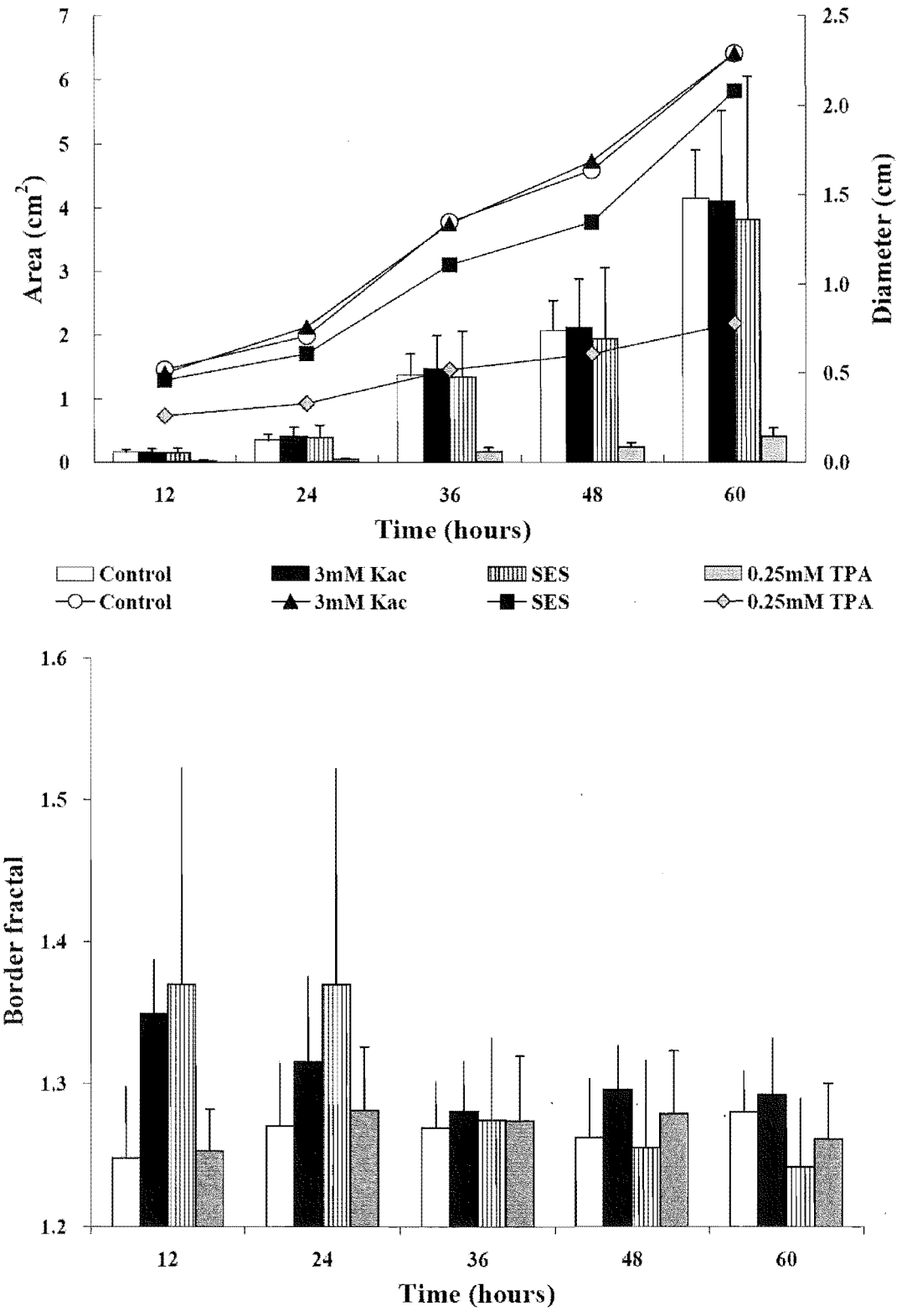


Figure 5.4 The effect of TPA on mycelial area (bar), radial extension (line) and border fractal dimension (bar) of Petri dish cultures.

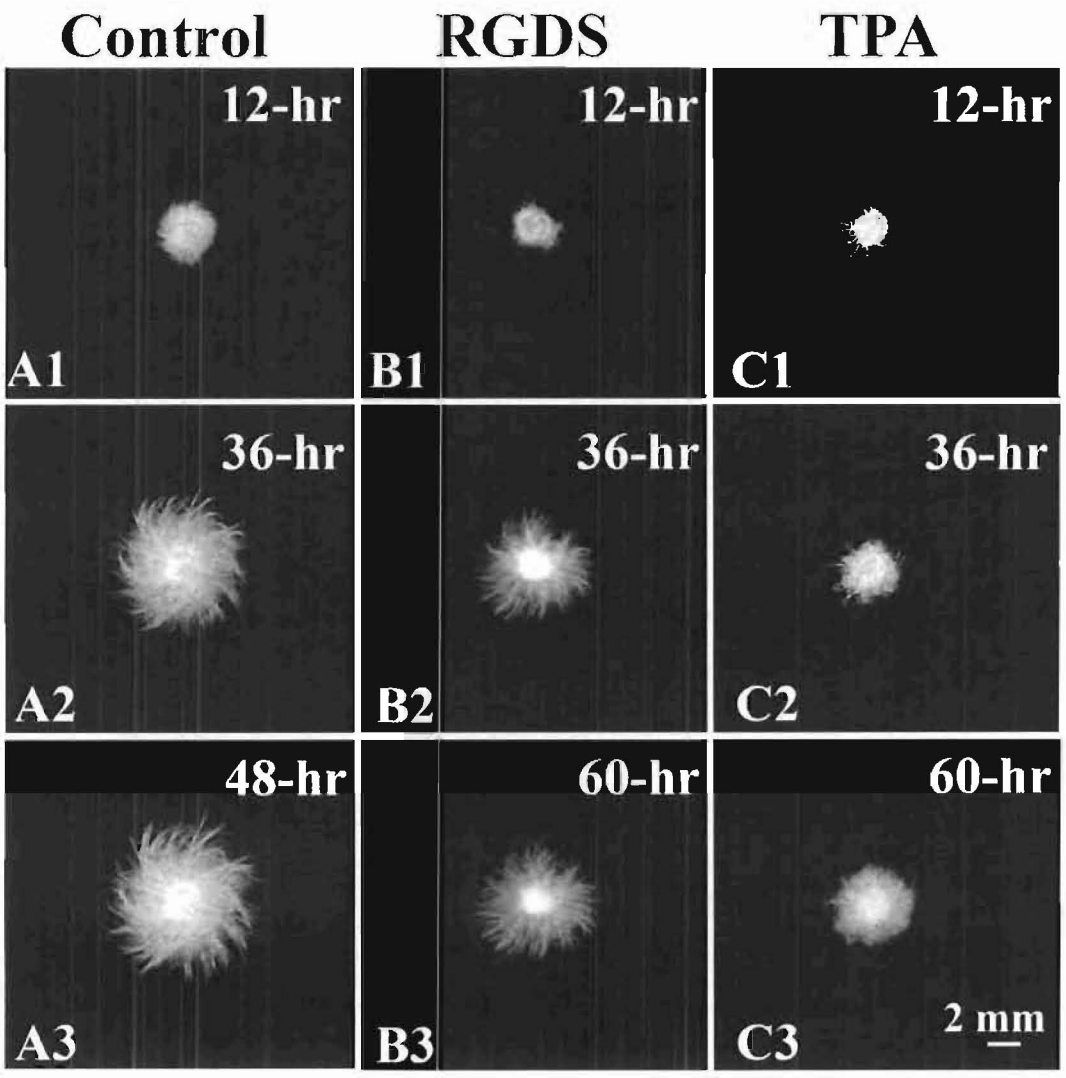


Figure 5.5 The effect of RGDS and TPA on the mycelial morphology of *A. bisexualis*, grown in a slide chamber.

A1-3 depicts control treatments, B1-3 RGDS and C1-3 TPA treatments. Times (in hrs) after inoculation are indicated.

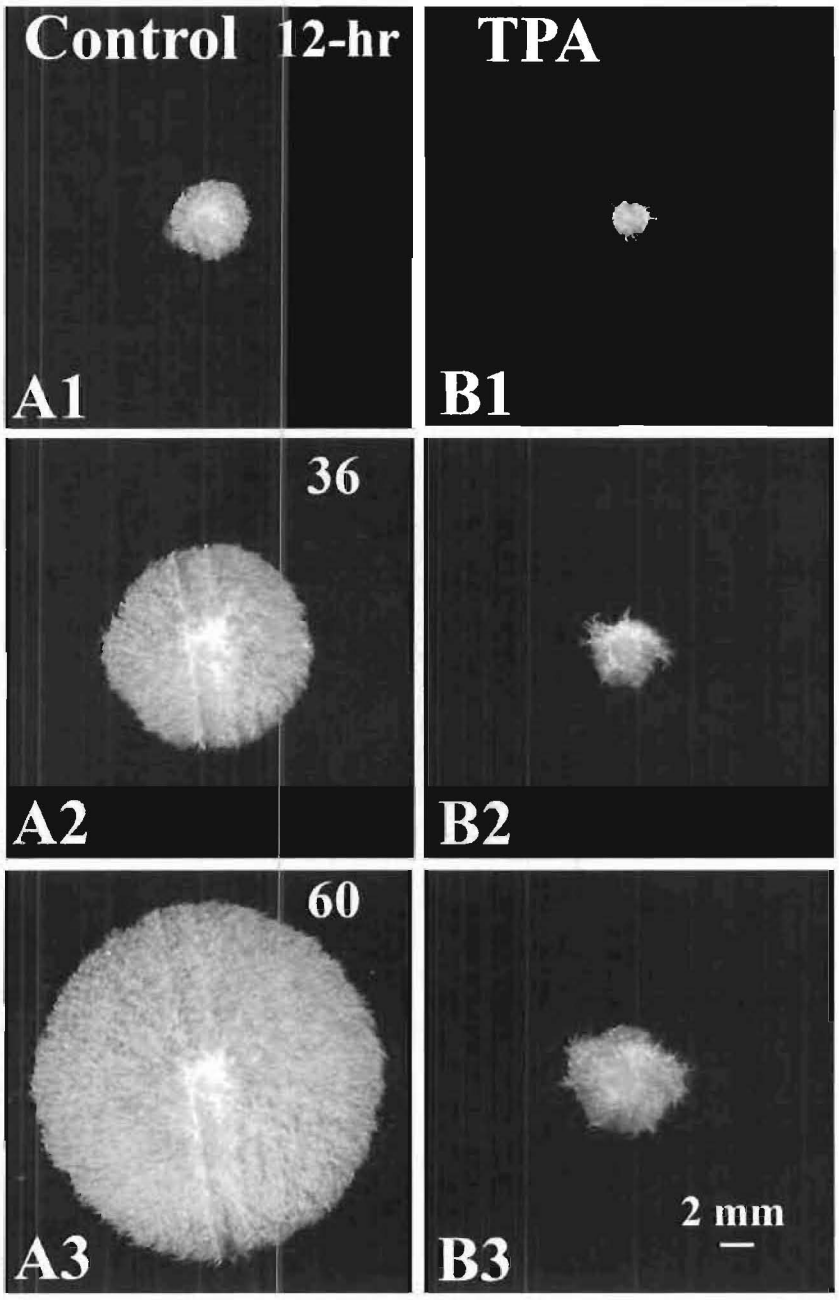


Figure 5.6 The effect of 0.25 mM TPA on mycelial morphology of *A. bisexualis*, grown in a Petri dish.

A control treatment is shown in A1-3 and TPA is shown in B1-3 with the time in hrs after inoculation indicated.

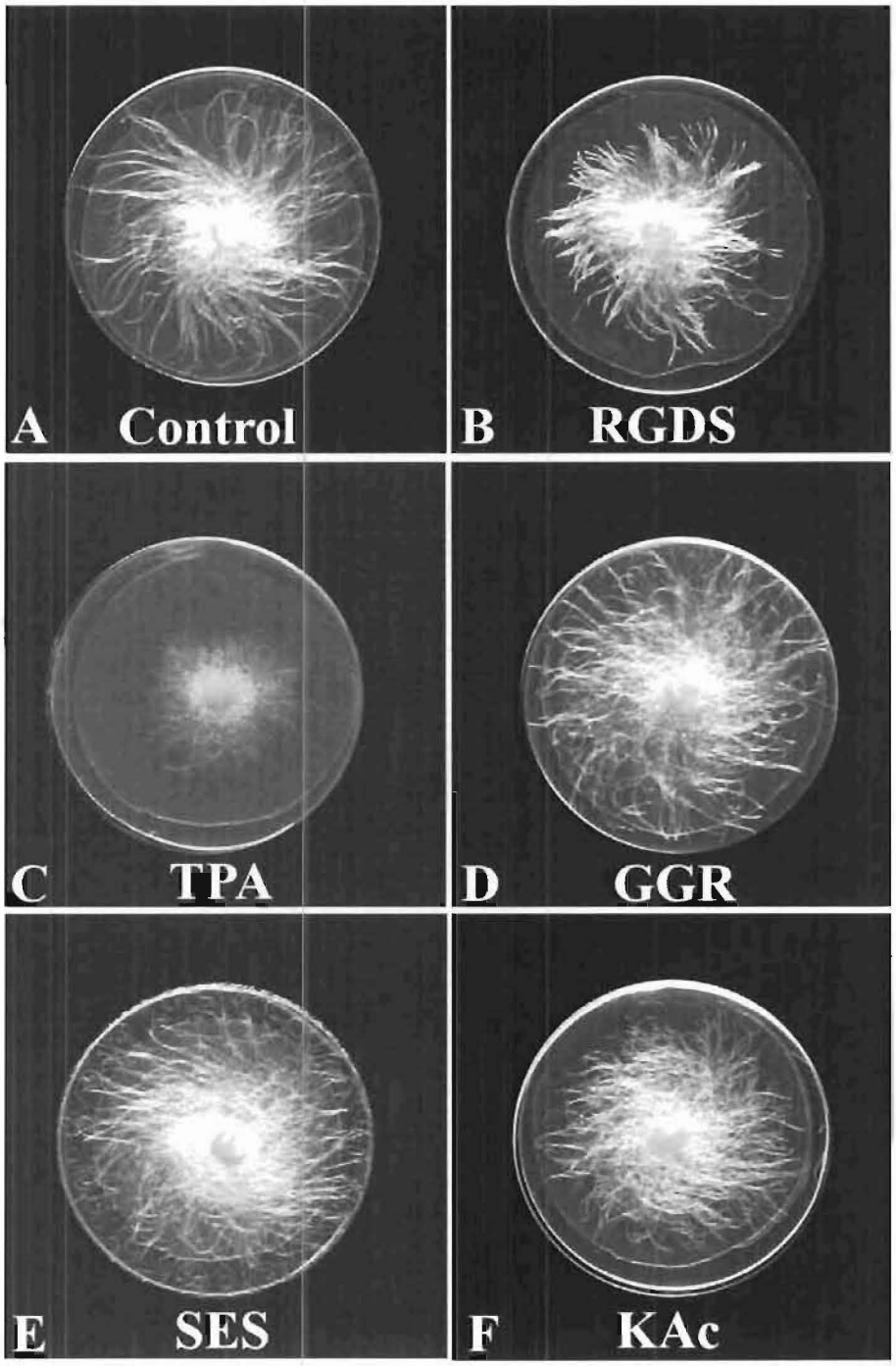


Figure 5.7 The mycelial morphology of *A. bisexualis* growing in the presence of different treatments in a slide chamber.

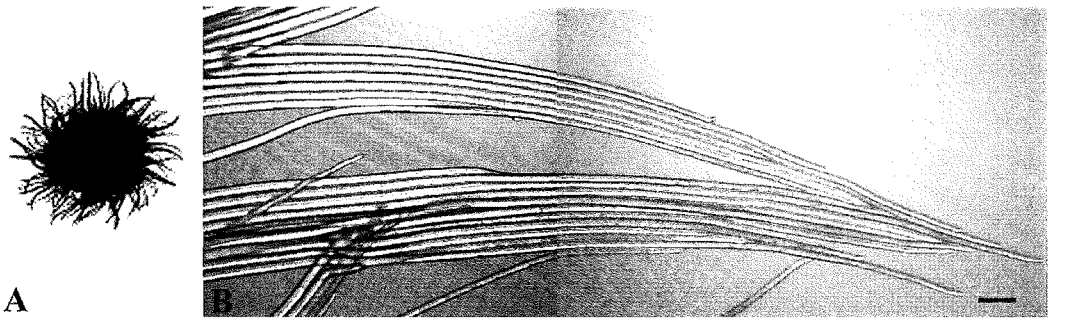


Figure 5.8 RGDS reduced branching and increased border fractal

A binarised mycelial image B unbranched hyphae at the edge of mycelium.

Chapter 6
General Discussion/Conclusions

The objectives of this thesis, as stated in the Introduction, were to investigate the effect of various chemical fixatives on the actin distribution in hyphae, to investigate the effect of RGD containing peptides on various aspects of hyphal morphology including cell wall-plasma membrane attachment sites and finally to investigate the effect of RGD containing peptides on the morphology of mycelia as determined using fractal analysis. These investigations were intended to help gain a better understanding of the role of F-actin and associated molecules in hyphal tip growth.

Hyphal tip growth is thought to result from a balance between regulated extensibility of the tip and a protrusive force that enables extension (Kaminskyj *et al.*, 1992a). Extensibility is likely to arise due to the relative abilities of the cell wall and also the F-actin cytoskeleton to resist a protrusive force. As such control of tip yielding may involve dynamic rearrangements of the F-actin cytoskeleton and fine control of the cross-linking of wall polymers. The protrusive force is often assumed to be turgor pressure, however, the relationship between turgor pressure and growth rate is complex which suggests that additional mechanisms of force generation may be present (Kaminskyj *et al.*, 1992a). Models have been proposed in which protrusion of the apical cytoplasm occurs by a mechanism similar to that of the extension of lamellipodia in animal cells. In these cells F-actin provides the protrusive force. Thus there is the possibility that F-actin plays a key role in tip growth through regulation of tip extensibility and the provision of a protrusive force. Actin may be multifunctional, however, as it is also thought to play a role in several other aspects of apical extension such as the process of cell wall synthesis, cytoplasmic migration, and the movement and positioning of organelles (Bachewich & Heath, 1998; Heath, 1990; Jackson & Heath,

1990a; Jackson & Heath, 1993). Peripheral F-actin networks may also influence the tip-high gradients of stretch activated channels in oomycete hyphae (Levina *et al.*, 1994).

In considering the roles of F-actin in tip growth an understanding of its location in the hypha is critical. In hyphal organisms, there are typically two patterns of F-actin observed: plaques and fibrils that sometimes associate to form cables. Usually the distribution of F-actin is peripheral. The actual distribution of plaques and cables of F-actin may differ among species, but as actin filaments are very labile, these variations in actin distribution may be due to different fixation methods used in the various studies (Heath, 1987). Heath (1987) described the actin distribution in chemically fixed hyphae of *Saprolegnia ferax*, at the tip there was a characteristic fibrillar cap with plaques and cables in the more subapical regions. This contrasts with other tip growing cells such as pollen tubes and root hairs, however, in which an actin clear zone is present at the extreme tips of cells which full of vesicles. This is thought to be an area where actin is dynamically arranged into microfilaments and that this imparts polarity on the cell.

Studies of actin distribution have used several different methods to fix and stain the hyphae. In any such study, it is important to preserve the structural organization of the F-actin as close as possible to that of the living cells. Possibly the best way is to introduce a label into living cells, which upon subsequent observation should reveal the dynamic nature of the F-actin. The only such report on oomycetes used the technique of electroporation to introduce fluorescently labeled rhodamine Phalloidin into growing hyphae. This showed an actin depleted zone at the tip, but a lack of brightly stained fibrils and plaques in subapical regions (Jackson & Heath, 1990b). This led to the suggestion that not all populations of F-actin were detected in this way and thus that the F-actin depleted zone was possibly an artefact. Because of this problem, hyphae have tended to be fixed by using either chemical or rapid freeze fixation rather than using electroporation.

As described in Chapter 2, in the present study the combination of methylglyoxal and formaldehyde in a chemical fixative gave a similar overall F-actin pattern and an additional structural component, the F-actin depleted zone was visible. This suggests that the previous observations on live, electroporated hyphae (Jackson & Heath, 1990b) may not have been artifactual. It is suggested, that the ability in the present study to

observe this structure is due to the improved quality of the combination fixative. The advantages of this combination fixative, relative to the commonly used fixative of formaldehyde alone or a combination of formaldehyde and glutaraldehyde are as follows. Firstly the fixation and subsequent staining is much more consistent with the combination. Secondly, a higher concentration of actin filaments remained in these cells, following fixation. Thirdly, the cross-linking of the filamentous actin network was better with the combination fixative and finally no bulk movements of cytoplasm were observed upon application of the fixative. In previous studies bulk movements of cytoplasm were observed as fixative was applied (Kaminskyj *et al.*, 1992b), which may have caused actin to move into F-actin depleted zones, thereby masking their existence. Evidently if these movements do not occur, as in our study, then there is less potential for such fine structural details to be lost.

The improvement of fixation may be due to the fact that the structures of methylglyoxal and glutaraldehyde are similar enough to enable irreversible cross-linking, yet different enough that methylglyoxal negates the problems of auto fluorescence. Other techniques such as rapid freeze fixation and the use of green fluorescent protein in living cells has shown limited detectable F-actin in the tips of other tip growing cells (Geitmann & Emons, 2000; Vidali & Helper, 2001). It has been suggested that these may represent areas of delicate, unstable F-actin, such as those described in pollen tubes (Gibbon *et al.*, 1999) and in the tips of algal rhizoids (Hable & Kropf, 1998). Such a depleted zone has been suggested to impart morphological polarity on cells (Vidali & Helper, 2001) but it is also possible that in oomycetes areas of discontinuity in the F-actin cap are indicative of areas where greater tip extensibility is possible.

For tip growth to occur wall vesicles need to move to, and be exocytosed at, the tip. This process would require a discontinuity in any peripheral actin network and it is suggested that the F-actin depleted zone may represent such an area. If the actin network were resisting turgor then it is possible that this depleted area could give rise to a burst-prone hole in the make up of the tip. Heath (1995) has suggested this problem could be overcome if the vesicles were coated with actin, although a discontinuity in the peripheral actin network, as described in Chapter 2, does not support this argument. An alternative is suggested in which an actin-depleted zone may present a means to allow

the internal determination (and hence regulation) of an area that would more readily yield to turgor pressure or, alternatively, a protrusive force. This would also presumably be the major site of exocytosis of wall material and it is interesting to note the recent finding of Ketelaar *et al.*, 2003 in root hairs that localised instability of the actin network can determine the site of exocytosis.

As oomycetes have been shown to grow in the absence of measurable turgor there have also been arguments that actin may provide a protrusive force at the tips of hyphae in a manner similar to protrusion of pseudopodia/lamelliposia in animal cells. Such protrusive forces in animal cells are thought to arise from the insertional polymerization of actin microfilaments (Pantaloni *et al.*, 2001). Such a mechanism would necessitate the existence of cytoskeletal cell wall linkages similar to those of animal cells and thus molecules similar to the integrins may be present and involved in tip growth.

Integrins mediate the interaction of animal cells with the surrounding ECM faciliating cell adhesion, a process that is essential for anchorage. Such adhesions act as cues for cell migration and signals for growth and differentiation (Etzioni, 1999; Hynes, 1992). They also provide a means for F-actin to provide protrusive forces. With the provision of any force there is going to be an equal and opposite reaction. Thus, if actin pushed at the tip of a cell, there needs to be some means of resisting the equal and opposite reaction that would tend to push that actin back towards subapical regions. An adhesion that linked F-actin to the cell wall could enable such resistance.

In walled cells, such adhesions, between the plasma membrane and cell wall, which may be thought of as analogous to the ECM, have been reported. When such cells are plasmolysed, some cytoplasm and plasma membrane may remain attached to the cell wall (Attree & Sheffield, 1985; Henry *et al.*, 1996; Kagawa *et al.*, 1992; Kaminskyj & Heath, 1995; Oparka, 1994). This has led to the suggestion that these sites represent areas where integrins or integrin-like molecules are linking the F-actin cytoskeleton to the cell wall. In such cells a number of responses including pertubations of cell wall/plasma membrane adhesion sites, growth, development and differentiation have been observed upon addition of RGD-containing peptides. These peptides disrupt integrin ECM interactions in animal cells. These observations along with immunocytochemical data and limited sequence similarity have been used to argue that

proteins similar to the integrins are present in plants, fungi and oomycetes (Henry *et al.*, 1996; Schindler *et al.*, 1989).

The present study indicates that integrin-like proteins or at the very least RGDS-sensitive proteins are present in the oomycete *A. bisexualis*. That this peptide affected growth, as described in Chapter 3, suggests that these proteins may play a role in tip growth. Such a role may include the provision of cell wall-plasma membrane attachments as evidenced by the observations on retracting protoplasm and the presence of F-actin in these attachments, presented in Chapter 4. The F-actin cytoskeleton may provide a cytoplasmic continuum of these attachments as RGDS was found to disrupt the organization of F-actin. All of these observations are consistent with an apparatus that would enable F-actin to provide a protrusive force. This is clearly an area that is ripe for further research as these ideas are at present rather speculative and the confines of time prevented further investigation in the present study. One possibility would be to investigate the presence of these proteins in situations in the oomycetes where turgor is very low and thus where one might predict that F-actin might provide a protrusive force. With this in mind it is interesting to note that it has recently been found that the oomycetes are unable to regulate their turgor (Lew *et al.*, 2004). This is in contrast to the ascomycete *Neurospora crassa* which is able to regulate turgor. Thus turgidity in the oomycetes is not set by the organism but solely by the external environment. The study of Lew *et al.*, (2004) suggests that this argues against a universal mechanism of hyphal growth that is driven by turgor pressure.

Oomycetes grow as radially extending tubular hyphae that branch and interweave to form a mycelium. The morphology of a mycelium is therefore dependent upon a number of factors that include the rate and the direction of tip growth, the branching frequency and the angle at which the branch forms. The data presented in Chapter 5 indicate that RGDS can significantly decrease mycelial area and radial extension but has no apparent effect on the morphology at the edge of the mycelia as measured using the border fractal. This suggests that RGDS will affect the rate of tip growth but does not significantly affect the relationship between tip growth and branching. This is of interest in that many agents that slow tip growth will also increase the rate of branching (Jackson *et al.*, 2001). Therefore RGDS acts in a manner that differs from other agents

that simply slow growth. This suggests that the target of RGDS, integrin-like proteins, may be important with regard to regulating the relationship between tip growth and branching, in addition to playing a role in just the tip growth process itself.

How might these proteins play a role in branching? The relationship between tip growth and branching could be affected if perturbation of the arrangement of the F-actin cytoskeleton affected the hypha's perception of, and ability to control, the hyphal growth unit, which has been suggested as a cue for branching. The proteins may also control the arrangement of F-actin in the correct pattern that would enable bump formation which is an early process in the branching mechanism (Jackson *et al.*, 2001). This then raises the possibility that the integrin-like proteins themselves may act as morphogens, molecules that that indicate the location of a branch. It is also possible that RGDS will affect other proposed morphogens such as Ca^{2+} and transcellular ion currents. The distribution of ion channels and possibly other membrane transport proteins have been shown to be affected by agents that perturb F-actin distribution (Levina *et al.*, 1994).

In summary, this thesis presents evidence for the existence of an F-actin depleted zone in the apical F-actin cap of the oomycete *Achlya bisexualis*. Evidence is also presented for integrin-like proteins, or at the very least RGDS-sensitive proteins that likely interact with F-actin and play a role in the process of tip growth and branching.

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